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(71) Applicant (for all designated States except US): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants (for US only): MORGANTE, Michele [IT/IT]; Via Selletto, 224, I-33100 Udine (IT). VOGEL, Julie, Marie [US/US]; 1325 Summerhill Drive, Malvern, PA 19355-8717 (US).

(74) Agents: FLOYD, Linda, A. et al.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

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(54) Title: COMPOUND MICROSATELLITE PRIMERS FOR THE DETECTION OF GENETIC POLYMORPHISMS

(57) Abstract

Compound microsatellites used as self-anchoring primers for screening genomes to detect polymorphisms are provided. The preferred compound microsatellite primers are perfect compound dinucleotide primers wherein the adjacent dinucleotide pairs contain a common nucleotide which is in-phase across the compound junction. In a useful embodiment, these in-phase compound primers have been adapted to other known screening assays including variations of the AFLP assays.

TITLE
COMPOUND MICROSATELLITE PRIMERS FOR THE
DETECTION OF GENETIC POLYMORPHISMS
FIELD OF INVENTION

5   The present invention relates the use of perfect,
compound simple sequence repeats (SSR) as self-
anchoring primers for the identification and analysis
of DNA sequence polymorphisms. More specifically it
has been observed that any one type of simple sequence
10 repeat (SSR) in both plant and animal genomes often
exists directly adjacent to an SSR of a different type,
usually with perfect periodicity of one of the
component nucleotides shared by both SSRs. This
observation has allowed the design of self-anchoring
15 primers in new variations of polymerase chain reaction-
based multiplexed genome assays, including inter-repeat
amplification and amplified fragment length
polymorphism assays. These method variations
collectively have been termed selective amplification
20 of microsatellite polymorphic loci (SAMPL).

BACKGROUND
The ability to map eukaryotic genomes has become
an essential tool for the diagnosis of genetic
diseases, and for plant breeding and forensic medicine.
25 An absolute requirement for elucidation of any genetic
linkage map is the ability to identify DNA sequence
variation. The realization that genetic (DNA)
polymorphisms between phenotypically identical
individuals are present and can be used as markers for
30 genetic mapping has produced major advances in the art
of developing eukaryotic linkage maps.
      Techniques for identifying genetic polymorphisms
are relatively few and to date have been time consuming
and labor intensive. One of the most common techniques
35 is referred to as restriction fragment length

polymorphism or RFLP (Botstein et al. *Am. J. Hum. Genet.* 342, 314, (1980)). Using RFLP technology, genetic markers based on single or multiple point mutations in the genome may be detected by

5   differentiating DNA banding patterns from restriction enzyme analysis. As restriction enzymes cut DNA at specific target site sequences, a point mutation within this site may result in the loss or gain of a recognition site, giving rise in that genomic region to

10   restriction fragments of different length. Mutations caused by the insertion, deletion or inversion of DNA stretches will also lead to a length variation of DNA restriction fragments. Genomic restriction fragments of different lengths between genotypes can be detected

15   with region-specific probes on Southern blots (Southern, E. M., *J. Mol. Biol.* 98, 503, (1975). The genomic DNA is typically digested with nearly any restriction enzyme of choice. The resulting fragments are electrophoretically size-separated, transferred to

20   a membrane, and then hybridized against a suitably labelled probe for detection of fragments corresponding to a specific region of the genome. RFLP genetic markers are particularly useful in detecting genetic variation in phenotypically silent mutations and serve

25   as highly accurate diagnostic tools. RFLP analysis is a useful tool in the generation of codominant genetic markers but suffers from the need to separate restriction fragments electrophoretically and often requires a great deal of optimization to achieve useful

30   background to signal ratios where significant polymorphic markers can be detected. In addition, the RFLP method relies on DNA polymorphisms existing within actual restriction sites. Any other point mutations in the genome usually go undetected. This is a

35   particularly difficult problem when assaying genomes

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with inherently low levels of DNA polymorphism. Thus, RFLP differences often are difficult to identify.

Another method of identifying polymorphic genetic markers employs DNA amplification using short primers

5   of arbitrary sequence. These primers have been termed 'random amplified polymorphic DNA', or "RAPD" primers, Williams et al., *Nucl. Acids. Res.*, 18, 6531 (1990) and U.S. 5,126,239; (also EPO 543 484 A2, WO 92/07095, WO 92/07948, WO 92/14844, and WO 92/03567). The RAPD

10 method amplifies either double or single stranded nontargeted, arbitrary DNA sequences using standard amplification buffers, dATP, dCTP, dGTP and TTP nucleotides, and a thermostable DNA polymerase such as *Taq* polymerase. The nucleotide sequence of the primers

15 is typically about 9 to 13 bases in length, between 50 and 80% G+C in composition and contains no palindromic sequences. Differences as small as single nucleotides between genomes can affect the RAPD primer's binding/target site, and a PCR product may be generated

20 from one genome but not from another. RAPD detection of genetic polymorphisms represents an advance over RFLP in that it is less time consuming, more informative, and readily adaptable to automation. The use of the RAPD assay is limited, however, in that only

25 dominant polymorphisms can be detected; this method does not offer the ability to examine simultaneously all the alleles at a locus in a population. Nevertheless, because of its sensitivity for the detection of polymorphisms, RAPD analysis and

30 variations based on RAPD/PCR methods have become the methods of choice for analyzing genetic variation within species or closely related genera, both in the animal and plant kingdoms.

A third method more recently introduced for

35 identifying and mapping genetic polymorphisms is termed

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amplified fragment length polymorphism or AFLP
(M. Zabeau, EP 534,858). AFLP is similar in concept to
RFLP in that restriction enzymes are used to
specifically digest the genomic DNA to be analyzed.

5   The primary difference between these two methods is
that the amplified restriction fragments produced in
AFLP are modified by the addition of specific, known
adaptor sequences which serve as the target sites for
PCR amplification with adaptor-directed primers.

10   Briefly, restriction fragments are generated from
genomic DNA by complete digestion with a single or
double restriction enzyme combination, the latter using
a "frequent" cutter combined with a "rare" cutter.
Optimal results are obtained when one of these enzymes

15   has a tetranucleotide recognition site, and the other
enzyme a hexanucleotide site. Such a double enzyme
digestion generates a mixture of single- and double-
digested genomic DNA fragments. Next, double-stranded
adaptors composed of synthetic oligonucleotides of

20   moderate length (10-30 bases) are specifically ligated
to the ends of the restriction fragments. The
individual adaptors corresponding to the different
restriction sites all carry distinct DNA sequences.

       One of the adaptors, usually the one corresponding
25   to the hexanucleotide-site restriction enzyme, carries
a biotin moiety. The application of biotin-
streptavidin capture methodology leads to the selective
removal of all nonbiotinylated restriction fragments
(those bordered at both ends by the tetranucleotide

30   restriction site), and thus effectively enriches the
population for fragments carrying the biotinylated
adaptor at one or both ends. As a result, the DNA
fragment mixture is also enriched for asymmetric
fragments, those carrying a different restriction site

35   at each end. The selected fragments serve as templates

for PCR amplification using oligonucleotide primers
that correspond to the adaptor/restriction site
sequences. These adaptor-directed primers also can
include at their 3' ends from 1 to 10 arbitrary
5   nucleotides, which will anneal to and prime from the
genomic sequence directly adjacent to the restriction
site on the DNA fragment.

A PCR reaction using this type of pooled fragment
template and adaptor-directed primers results in the
10 co-amplification of multiple genomic fragments. Any
DNA sequence differences between genomes in the region
of the restriction sites or the 1-10 nucleotides
directly adjacent to the restriction sites leads to
differences, or polymorphisms (dominant and
15 codominant), in the PCR products generated. Multiple
fragments are simultaneously co-amplified, and some
proportion of these will be polymorphic between
genomes.

A fourth method of assaying polymorphisms has
20 involved utilizing the high degree of length variation
resulting from certain repeating nucleotide sequences
found in most genomes. Most if not all eukaryotic
genomes are populated with repeating base sequences
variously termed simple sequence repeats (SSR), simple
25 sequence length polymorphisms (SSLP), dinucleotide,
trinucleotide, tetranucleotide, or pentanucleotide
repeats, and microsatellites. Simple sequence repeats
have been demonstrated to be useful as genetic markers
(DE 38 34 636 A, Jackle et al.; Weber, et al, *Am J Hum
30 Genet* 44, 388, (1989); Litt et al., *Am J Hum* Genet 4,
397, (1989)). Weber et al., (*Genomics* 11, 695, (1991))
have successfully used SSRs for comparative analysis
and mapping of mammalian genomes, and several groups
(Akkaya, et al., *Genetics* 132, 1131 (1992), Morgante &
35 Olivieri, *Plant J* 3, 175 (1993), Wu & Tanksley, *Mol.*

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Gen. Genet. 241, 225, (1993)) have demonstrated similar
results with plant genomes. SSR polymorphisms can be
detected by PCR using minute amounts of genomic DNA
and, unlike RAPDs, they provide codominant markers and

5   can detect a high degree of genetic polymorphism
(Weber, J. L. (1990) In Tilghman, S., Daves, K., (eds)
'Genome Analysis vol. 1: Genetic and physical
mapping', Cold Spring Harbor Laboratory Press,
pp 159-181.).

10   Although SSR-directed PCR primers are highly
effective for detecting polymorphism, their use suffers
from a variety of practical drawbacks. Typically,
markers generated by these methods are obtained by
first constructing a genomic library, screening the

15   library with probes representing the core elements of a
particular repeat sequence, purifying and sequencing
the positive clones, and synthesizing the primers
specific for the flanking sequences for each cloned SSR
locus. Genomic DNA is then amplified to screen for

20   polymorphisms, and mapping of the genome is then
carried out. The entire process is time consuming,
expensive and technically demanding, and as a result
has been somewhat limited in its application.

         At least one method has been developed as an
25   attempt to circumvent these limitations and allow the
use of polymorphic SSR markers more directly, with no
a priori knowledge of particular SSR locus sequences.
Zietkiewicz, et al., (Genomics, 20, 176, (1994)), for
example, demonstrate that a single-primer PCR

30   amplification can be used to detect length
polymorphisms between adjacent $(CA)_n$ repeats in animal
and plant genomes. The PCR primers used for this assay
each contain a particular SSR sequence that is flanked
immediately 5' or 3' by 2 to 4 nucleotides of known or

35   arbitrary sequence; these anchor sequences anneal to

the non-SSR genomic sequences that flank the SSR
sequence in the genome and serve to "anchor" the primer
to a single position at each matching SSR locus.
Radiolabeled SSR-to-SSR amplification products,
5   generated when adjacent SSR sequences are oppositely
oriented and spaced closely enough in the genome, are
analysed by gel electrophoresis followed by
autoradiography. This approach eliminates the need for
cloning and sequencing SSRs from the genome, and
10   reveals an enriched polymorphic banding pattern
relative to single-locus SSR. Zietkiewicz et al,
attribute the enriched pattern to use of the arbitrary
sequence anchor, which allows the SSR primer to anneal
and prime from many SSR target loci simultaneously.
15   In a concept similar to Zetkiewicz, Wu et al.,
(Nucleic Acids Res. 22, 3257, (1994)) teach a method
for the detection of polymorphisms where genomic DNA is
amplified by asymmetric thermally cycled PCR using
radiolabeled 5' anchored primers consisting of
20   microsatellite repeats in the presence of RAPD primers
of arbitrary sequence. The method of Wu et al. is
useful for the generation of genetic markers that
incorporate many features of microsatellite repeats.
Wu et al. does not disclose the use of compound
25   microsatellite repeate primers for amplification.
          Simple sequence repeats may be classified in
several ways. In categorizing and characterizing human
$(CA)_n$ or $(GT)_n$ repeats, Weber, J. L. (Genome Analysis
Vol. 1, 159, 1990, Cold Spring harbor Laboratory Press,
30   NY) defines at least three types of $(CA)_n$ SSR: simple
perfect SSR, simple imperfect SSR, and compound SSR.
Each perfect SSR is considered to be a simple $(CA)_n$
tandem sequence, with no interruptions within the
repeat. Imperfect SSR are defined as those repeating
35   sequences with one or more interruptions of up to 3

nonrepeat bases within the run of the repeat. Compound
SSR are defined as those sequences with a CA or GT
repeat stretch adjacent to or within 3 nucleotides of a
block of short tandem repeats of a different sequence.

5 Weber notes that perfect sequence repeats in humans
comprise about 65% of the total $(dC-dA)_n(dG-dT)_n$
sequences cloned from the genome, imperfect repeats
about 25%, and compound repeats about 10%. Weber
theorizes that because perfect repeats contain the

10 longest uninterrupted repeat blocks, they appear to
provide the most useful information. Weber also
teaches that repeats composed of 12 or more
uninterrupted units are consistently more polymorphic
than are shorter repeat stretches. Because imperfect

15 repeats generally contain shorter repeat stretches,
they appear to be less useful as indicators of
polymorphism. Compound repeats in general have not
been well characterized, and their potential
informativeness has not been clearly established.

20 Others have used the polymorphisms detectable
within perfect, imperfect and compound SSR loci to
build genetic linkage maps. Buchanan et al. (*Mammalian
Genome*, 4, 258, (1993)) teach that there is little
difference in the utility of the different SSR types in

25 the ovine genome with respect to their absolute
polymorphism levels; the perfect, imperfect and
compound repeats although likely present in the genome
at differing frequencies (perfect and imperfect simple
SSR's are more frequent than compound) were found to

30 have similar average Polymorphism Information Content
(PIC) values as defined by Botstein et al. (*Am. J. Hum.
Genet.*, 32, 314, (1980)). In a study of $(GT)_n$ SSR in
the Atlantic salmon genome, Slettan et al.(*Animal
Genetics*, 24, 195, (1993)) found both perfect and

35 imperfect simple SSR but no compound repeats. In an

examination of the equine genome, Ellegren et al.(*Amimal Genetics*, 23, 133, (1993)) identified the highest levels of polymorphism involving $(TG)_n$ and $(TC)_n$ repeats among horse genotypes using primers

5 designed to amplify perfect or imperfect simple repeats; although two of eight cloned $(GT)_n$ repeats were identified to be compound in structure (one perfect, one imperfect), neither was characterized further. Condit & Hubbell (*Genome* 34, 66 (1991)), in

10 characterizing large-insert clones carrying $(AC)_n$ and $(AG)_n$ repeats from tropical trees and maize, found that 10-20% of inserts carrying one type of repeat also carried the other, and that many $(AC)_n$ sites also had other two-base repeats adjacent or nearby. Finally,

15 Browne et al. (*Nucl. Acids Res.*, 20, 141, (1991)), in an attempt to characterize $(CA)_n$ SSR sequences in the human genome by DNA sequencing with degenerate $(CA)_n$ primers, disclose that 88% of their $(CA)_n$ repeats carried AT base pairs at one or both ends of the CA

20 repeat.

To date, the record in the literature would indicate that although it varies with each type of genome, the incidence of compound SSRs in a genome is lower than that of either perfect of imperfect simple

25 SSR sequences. Nevertheless, the information content (PIC value) of compound SSR sequences has been shown to be generally high. In addition, the literature would indicate that the detection of genetic polymorphisms by way of specifically isolating compound SSR loci

30 generally would have marginal success; the use of probes or primers designed to recognize and thus specifically isolate individual compound SSR loci would be less efficient for generating large numbers of new SSR markers as compared to the isolation of the more

35 numerous simple SSR sequences. Applicants have,

however, unexpectedly discovered that compound SSR's, particularly those containing $(AT)_n$ repeats are highly polymorphic in eukaryotic genomes, and that oligo-nucleotides designed to anneal specifically to a

5 specific type of SSR, termed herein as a perfect in-phase compound SSR, are particularly useful in the generation and detection of polymorphisms between eukaryotic genomes. A "perfect" compound SSR is one in which two different repeating sequences, each of which

10 could be composed of di-, tri-, tetra-, or penta-nucleotide units, are located very near each other, with no more than 3 intervening bases between the two repeat blocks. One category of perfect compound repeat is one in which the two constituent repeats are

15 immediately adjacent to one another, with no intervening bases. Further, a perfect compound SSR can be classified to be "in-phase" if both of the component simple repeats share a common nucleotide whose spacing is conserved across the repeat junction and over the

20 length of the two repeat blocks. For example, the in-phase perfect compound SSR, $(AT)_n(AG)_n$, maintains the adenosine base "in-phase" across both components of this perfect compound structure.

Applicants have discovered that in-phase perfect

25 compound SSR sequences such as $(dC-dA)_n(dT-dA)_n$ are abundant in both animal and plant genomes. Although the frequency of occurrence of each type of perfect compound SSR sequence varies within, as well as between, species, those sequences that are in-phase are

30 of sufficiently high frequency in all eukaryotic genomes examined, and appear to be both well dispersed and highly polymorphic. Based upon their observation that the junction spanned by such directly adjacent, in-phase perfect repeats is absolutely predictable,

35 Applicants have developed methodology which utilizes

synthetic oligonucleotides containing in-phase compound
sequences as self-anchoring primers in new variations
of polymerase chain reaction-based multiplexed genome
assays, including inter-repeat amplification and
5   amplified fragment length polymorphism assays.
Applicants have found that the 5' end of the compound
SSR primer serves as an extremely efficient anchor base
for primer extension that occurs from the 3'-end
repeat. This primer extension initiates from inside
10   the compound SSR target sequence, such that any length
variation between different alleles at a target SSR
locus is detectable as a corresponding length bariation
in the resulting amplification products. Because such
use of perfect compound SSRs as amplification primers
15   generates multiple products wherein a high proportion
are polymorphic (as high as 80%), Applicants believe
that the method of their invention greatly facilitates
the simultaneous identification of multiple genomic
polymorphisms, both codominant and dominant. Thus, the
20   present method offers great advantage in identifying
polymorphic markers linked to genetic traits of
interest, and also offers an efficient and convenient
generic technique for genome fingerprinting and whole-
genome comparisons.

25   SUMMARY OF THE INVENTION
        This invention provides an improved method of
detecting polymorphisms between two individual nucleic
acid samples comprising amplifying segments of nucleic
acid from each sample using primer-directed
30   amplification and comparing the amplified segments to
detect differences, the improvement comprising wherein
at least one of the primers used in said amplification
consists of a perfect compound simple sequence repeat.
In a preferred embodiment, the compound primer is in-
35   phase.

In a most preferred embodiment the present invention provides a method for the detection of genetic polymorphisms using a combination of in-phase perfect compound SSR primers and synthetic adaptor-
5   directed primers for PCR amplification from restriction enzyme digested genomic DNA templates to which fixed-sequence adaptors have been ligated.

The present methods are particularly useful in the areas of clinical genetic diagnostics, forensic
10 medicine (where it is important to detect small polymorphic changes in nucleic acid composition), as well as in the areas of animal and plant breeding and gene mapping. As specific applications, these methods have great utility for genome fingerprinting,
15 polymorphic marker identification (i.e., "marking" a phenotypic trait), and germplasm comparisons.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 illustrates a schematic representation of SSR-to-adaptor amplification. Panel a depicts the
20 restriction enzyme double digestion, adaptor ligation, and biotin-streptavidin selection process for generating the DNA template mixtures, as well as the selective nature of a specific SSR primer for exponential SSR-to-adaptor amplification from a complex
25 template DNA mixture. Panel b depicts the selective nature of the adaptor-directed primer, in this case containing at its 3' end one nondegenerate selective nucleotide, for discriminating template fragments that otherwise would be amplified by a common SSR primer.
30 Diagonally hatched boxes indicate the biotinylated adaptor corresponding to a restriction enzyme with a 6-bp recognition site, and dark boxes depict the nonbiotinylated adaptor corresponding to an enzyme with a 4-bp recognition site. The biotin moiety is
35 indicated by a solid circle. The vertically striped

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box indicates either a simple SSR or a compound SSR of
a particular type that matches the SSR primer used for
the PCR amplification. Arrows depict PCR primers, with
the arrowhead showing the direction of primer
5   extension; solid/dark arrows indicate adaptor-directed
PCR primers, and vertically striped-hatched arrows
indicate primers corresponding to the SSR sequence
depicted on the template fragments. Only the SSR-
directed primer is tagged with either a fluorescent or
10 radiolabel, as indicated by *. Pancel C depicts a
perfect, in-phase compound SSR as a double-stranded
locus in the genome ($(AT)_x(AG)_y$, here where x=11.5 and
y=10), which can serve as a target site for two classes
of primer, each representing one strand of the double-
15 stranded target locus. Individual primers within each
class can differ by the relative length of each
constituent repeat. The two classes of primer initiate
primer extension in opposite directions (small arrows).
In every case, the primer anneals to a fixed site at
20 the target, and primer extension initiates inside the
SSR region. In each case, any length variation in the
3'-most repeat between genomes could be detected as a
codominant polymorphism using SSR-to-adaptor
amplification.
25   Figure 2 illustrates an autoradiograph of a
denaturing polyacrylamide gel that compares the co-
amplified products from SSR-to-adaptor reactions on
Taq I + Pst I digested, adaptor modified, biotin-
selected template DNAs prepared from four different
30 *Glycine max* or *Glycine soja* genotypes (N, *max* N85-2176;
No, *max* NOIR-1; W, *max* wolverine; S, *soja* PI 81762).
^{33}P-labeled simple SSR primers containing 3 bp
degenerate anchors at their 5' ends [HBH(AG)$_{8.5}$,
DBD(AC)$_{7.5}$, HVH(TG) $_{7.5}$] are paired with unlabeled Taq I
35 adaptor-directed primers containing either zero

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(TaqAd.F) or one (Taq.pr6, Taq.pr8) selective
nucleotide at their 3' ends. Cold start amplifications
employed either a constant temperature (58°C) or
touchdown (59°C final temperature) thermocycle profile
5 (left and right panels, respectively). An arrow
indicates a likely codominant polymorphism.

Figures 3a and 3b illustrate autoradiographs of
denaturing polyacrylamide gels that compare the co-
amplified products from SSR-to-adaptor reactions on
10 Taq I + Pst I digested, adaptor-modified, biotin-
selected templated DNAs prepared from 15 different
soybean genotypes. ^{33}P-labeled primers corresponding
to perfect compound SSRs, $(TC)_{4.5}(TG)_{4.5}$,
$(CT)_{7.5}(AT)_{3.5}$ and $(CA)_{7.5}(TA)_{2.5}$ [panel a] or
15 $(TG)_{4.5}(AG)_{4.5}$ and $(TC)_{4.5}(AC)_{4.5}$ [panel b], each are
paired with unlabeled Taq I-adaptor primers containing
either zero (TaqAd.F) or one (Taq.pr8) 3'-selective
nucleotide, under cold start amplification conditions
utilizing a touchdown (56°C final) thermocycle profile.
20 In each set, lane 1, *Glycine max* wolverine; lane 2, *G.
max* NOIR-1; lane 3, *G. max* N85-21761; lane 4, *G. max*
Harrow; lane 5, *G. max* CNS; lane 6, *G. max* Manchu; lane
7, *G. max* Mandarin; lane 8, *G. max* Mukden; lane 9, *G.
max* Richland; lane 10, *G. max* Roanoke; lane 11, *G. max*
25 Tokyo; lane 12, *G. max* PI 54.610; lane 13, *G. max*
Bonus; lane 14, *G. soja* PI 81762; lane 15, *G. soja* PI
440.913.. The size distribution of products is similar
to that in Figure 2. Lane 9 of the
$(TG)_{4.5}(AG)_{4.5}$+Taq.Pr8 set is a misloading of an
30 incorrect (non-soybean) sample.

Figure 4 illustrates an autoradiograph of a
denaturing polyacrylamide gel that compares the co-
amplified products from SSR-to-adaptor amplifications
performed on five different soybean genotypes (lane 1,
35 *G. max* wolverine; 2, *G. max* NOIR-1; 3, *G. max* N85-2176;

4, *G. max* Bonus; 5, *G. soja* PI 81762) prepared by
digestion with Taq I combined with either Hind III or
Pst I (H+T or P+T respectively). Cold start
amplifications using a 56°C touchdown thermocycle
5   profile utilized the ^{33}P-labeled SSR primer,
$(CA)_{7.5}(TA)_{2.5}$, in combination with the indicated
unlabeled Taq I adaptor-directed primer, Taq Ad.F or
Taq.Pr8 (zero or one 3'-selective nucleotide,
respectively). X indicates a misloaded (incorrect)
10 lane.

Figure 5a illustrates an autoradiograph of a
denaturing polyacrylamide gel demonstrating the
segregation of polymorphic co-amplification products of
66 F2 progeny from a cross between *G. soja* PI 81762 and
15 *G. max* Bonus. A ^{33}P-labeled primer corresponding to
the perfect compound SSR, $(CA)_{7.5}(TA)_{2.5}$, was paired
with the 3'-selective nucleotide Taq.pr6 adaptor-
directed primers. Blank lanes are the result of
"missing data". The scored polymorphic bands that
20 segregate in this population are indicated. B, bonus
parent; S, *soja* PI81762 parent. Figure 5b illustrates
the map positions of 6 of the polymorphic segregating
amplification products contained in panel a, as
determined by MAPMAKER analysis of the products'
25 respective segregation scores (contained in Table VI).

Figure 6a illustrates an autoradiograph of a
denaturing polyacrylamide gel comparing amplifications
using the perfect compound SSR primer $(CA)_{7.5}(TA)_{2.5}$,
paired with either the Taq.AdF or the more selective
30 Taq.pr8 adaptor-directed primer, on template DNAs
derived from either 5 (wolverine, NOIR-1, N85-2176,
Bonus, PI 81762) or 15 soybean cultivars same ordering
of genotypes as in Figures 3a and 4, respectively, and
6 mammalian individuals (one rat, four human, one mouse
35 BALB/C). All biotin-selected templates were prepared

using Taq I combined with either Hind III or Pst I.
Figure 6b illustrates a similar comparison of the co-
amplification products using $(CT)_{7.5}(AT)_2$ and
$(GA)_{7.5}(TA)_2$ perfect compound SSR primers, from Pst I +
5 Taq I prepared template DNAs. The size distribution of
products is similar to that in panel a. Figure 6c
illustrates the SSR-to-adaptor amplification products
generated from Taq I + Hind III prepared templates of
Zea mays (corn) and salmon DNA templates, in comparison
10 to those from soybean Bonus and PI81762, using
$(CA)_{7.5}(TA)_{2.5}$ paired with Taq.pr6 primer. Lane 1,
Z. mays B73; lanes 2 and 3, individual salmon sources;
lane 4, *Z. mays* CM27; lane 5, *Z. mays* T232; lane 6, *Z.
mays* DE811ASR; lane 7, *Z. mays* LH132; lanes 8-10, two
15 F2 individuals from a *G. max* Bonus x *G. soja* PI 81762
cross. The distribution of product sizes is similar to
that shown in Figure 6a.

Figure 7 illustrates an autoradiograph of a
denaturing polyacrylamide gel comparing the co-
20 amplification products from Taq I + Pst I prepared
soybean templates (S, *Soja* PI81762; W, Wolverine; B,
bonus) using $(CA)_{7.5}(TA)_{2.5}$ paired individually with
Taq I adaptor-directed primers carrying either zero
(Taq.AdF) or one specific 3'-selective nucleotide
25 (Taq.pr5, .pr6, .pr7, .pr8).

Figure 8 illustrates an autoradiograph of a
denaturing polyacrylamide gel comparing SSR-to-adaptor
amplification from wolverine and PI 81762 soybean
cultivars using SSR primers respresenting the
30 complementary strands of a perfect compound SSR double
stranded sequence $[(AT)_x(GT)_y:(CA)_x(TA)_y]$ paired
separately with three different Taq I adaptor-directed
primers, Taq.AdF, Taq.pr6 and Taq.pr8. Each strand of
the double stranded compound SSR sequence is
35 represented by three primers that differ by the

relative lengths of each of the two constituent repeats within the primer. The $(AT)_{8.5}(GT)_{2.5}$ and $(AT)_{6.5}(GT)_{4.5}$ primers were completely inefficient (no amplification products generated (data not shown) and

5    $(AT)_{3.5}(GT)_{6.5}$ was moderately successful (shown), in comparison to the three, more efficient $(CA)_x(TA)_y$ primer types.

Figure 9 illustrates autoradiographs of denaturing polyacrylamide gels that compare the co-amplification

10   products from soybean cultivars, wolverine and PI 81762, using cold start (left) and hot start (right) methods for the initiation of thermocycling. For both methods, the perfect compound SSR primer, $(CA)_{7.5}(TA)_{2.5}$, is paired with each of the three Taq I

15   adaptor directed primers indicated.

Figure 10a illustrates an autoradiograph of a denaturing polyacrylamide gel comparing the co-amplification products from soybean (B, Bonus; S, soja; PI81762) and corn (b, B73; c, CM37) cultivars amplified

20   using $DBD(AC)_{6.5}$ in combination with no 2nd adaptor primer or with Taq.pr8. These templates are in the form of either intact, undigested DNA or Taq I + Pst I digested DNA (P+T), as indicated.

Figure 10b illustrates autoradiographs of

25   denaturing polyacrylamide gels comparing the amplification products obtained using ^{33}P-labeled 5'-anchored simple SSR primers [$DBD(AC)_{7.5}$ and $HBH(AG)_{8.5}$] or perfect compound SSR primers [$(AT)_{3.5}(AG)_{7.5}$ and $(AT)_{3.5}(GT)_{6.5}$] in single-primer

30   amplifications (no adaptor primer used) from both undigested and Taq I + Pst I digested, biotin-selected template DNAs from soybean wolverine (W) and PI81762 (S) cultivars. Cold start amplifications used either constant temperature (58°C) or 56°C touchdown annealing

35   profiles.

Figure 11 is a schematic representation of the cloning and sequencing of a chosen SSR-to-adaptor amplification product, and its conversion into a defined, single-locus marker. A genomic restriction

5 fragment carrying the targeted SSR repeat is bordered at both ends by restriction site-specific adaptors, Ad$_A$ and Ad$_B$. This fragment serves as the template for PCR amplification using an SSR-directed primer and a primer corresponding to one of the adaptors. This

10 amplification product is purified and sequenced, and a locus-specific flanking primer (lsfp-1) is designed. This lsfp-1 primer then is paired with a primer corresponding to the other adaptor, for PCR amplification using the adaptor-modified restriction

15 fragment mixture as template. The specific product obtained is then isolated and sequenced, and a second primer (lsfp-2) corresponding to the unique flanking sequence on the other side of the SSR is designed. The lsfp-1 + lsfp-2 primer pair uniquely defines this SSR

20 locus, and can be used to amplify directly from genomic DNA to visualize SSR length polymorphism at this locus. Applicants have provided 89 sequence listings in conformity with 37 C.F.R. 1.821-1.825 and Appendices A and B ("Requirements for Application Disclosures

25 Containing Nucleotides and/or Amino Acid Sequences").

DETAILED DESCRIPTION OF THE INVENTION

As used herein the following terms may be used for interpretation of the claims and specification.

The term "Simple sequence repeat (SSR)" or

30 "microsatellite repeat (MS)" or "short tandem repeat" or "dinucleotide repeat" or "trinucleotide repeat" or "tetranucleotide repeat" or "microsatellite" or "simple sequence length polymorphisms" (SSLP) all refer to stretches of DNA consisting of tandemly repeating di-,

35 tri-, tetra-, or penta-nucleotide units. An SSR region

can be as short as two repeating units, but more
frequently is in excess of 8-10 repeating units.
Simple sequence repeats are common in virtually all
eukaryotic genomes studied and have been identified as
5   useful tools for the study of genetic polymorphisms.

Classification of SSR loci or SSR sequences as
used herein is based upon (but not identical to) the
definitions suggested by Weber (*Genomics* 7, 524 (1990))
for the categorization of human $(CA)_n$ dinucleotide
10   repeats.

The term "simple SSR" will refer to a region
comprised of at least three or more of the same
tandemly repeated di-, tri- or tetranucleotide
sequence, which is not adjacent in the genome to any
15   other different simple SSR. "Not adjacent" means not
closer than four nucleotides away on either side.

The term "compound SSR" refers to a region
consisting of two or more different simple SSR
sequences which are adjacent. "Adjacent" means that
20   differing simple SSR's are separated from one another
by three or fewer consecutive nonrepeat nucleotides.

The term "perfect SSR" refers to a simple SSR
wherein every simple repeating unit within the SSR is
intact and uninterrupted by nonrepeat nucleotides.

25   The term "imperfect SSR" refers to a simple SSR
wherein one or more of the constituent repeat units is
interrupted at least once within the SSR by three or
four consecutive nonrepeat nucleotides.

The term "perfect compound SSR" refers to a
30   compound SSR wherein the two constituent repeating SSR
regions are intact and uninterrupted by nonrepeat
bases, (i.e., they are perfect SSR's), and the two
perfect SSR regions are directly adjacent to one
another, having no intervening nucleotides.

The term "in-phase" refers to a potential feature of a perfect compound SSR wherein both constituent SSR regions share a common nucleotide that retains constant spacing spanning the junction of the two SSR regions.

5   The term "out of phase" refers to a potential feature of a perfect compound SSR wherein a nucleotide is common to the two or more constituent repeating regions, but it does not retain constant spacing or periodicity across the junction of the compound
10   structure.

The term "polymorphism" refers to a difference in DNA sequence between or among different genomes or individuals. Such differences can be detected when they occur within known or tagged genomic regions. A
15   "dominant polymorphism" is a DNA difference that is detectable only as the presence or absence of a specific DNA sequence at a single locus. Methods to detect dominant polymorphisms are able to detect only one allele of the locus at a time, and genomes
20   homozygous versus heterozygous for the detectable allele are indistinguished. A "codominant polymorphism" is a DNA difference at a locus between genomes whereby multiple alleles at the locus each can be distinguishable even when in heterozygous
25   combinations. Typically identifiable as mobility variants on electrophoretic gels, codominant polymorphisms can produce additive, nonparental genotypes when present in heterozygous form. A dominant polymorphism is most useful as a marker when
30   it is in coupling with the trait it marks, whereas a codominant polymorphism is equally useful when in coupling or in repulsion to a trait.

The term "touchdown amplification" or "touchdown PCR" will refer to a specific thermocycling profile for
35   the polymerase chain reaction whereby the annealing

temperature begins artificially high (or low) for the first few cycles, then is incrementally lowered (or raised) for a specified number of successive cycles until a final, desirable annealing temperature is

5   reached. The remaining cyles of the multiple cycle profile are then performed at this final, touchdown annealing temperature. Thermocycling using this strategy serves to reduce or circumvent spurious, nonspecific priming during the initial stages of gene

10 amplification, and imbalance between correct and spurious annealing is automatically minimized.

The terms "hot start" and "cold start" will refer to a general choice of methodologies for initiating thermocycling for a PCR amplification reaction. In a

15 cold start amplification, all the reaction components are assembled simultaneously at room temperature, prior to the first denaturation step. This approach allows for the possibility of spurious priming and nonspecific amplification products resulting from primer annealing

20 and primer extension at undesirably low temperatures. In contrast, a hot start approach employs the deliberate omission of at least one key component from the otherwise complete amplification reaction, thus preventing either primer annealing (if primer is

25 omitted) or extension (if either polymerase or nucleotides are omitted). After carrying out an initial high-temperature denaturation, the excluded component is added, and thermocycling proceeds. A hot start amplification thus serves to reduce or eliminate

30 the production of nonspecific products that result from spurious primer extension at nonstringent temperatures.

"Nucleic acid" refers to a molecule which can be single stranded or double stranded comprised of monomers (nucleotides) containing a sugar, phosphate

35 and either a purine or pyrimidine. In bacteria, lower

eukaryotes, and in higher animals and plants,
"deoxyribonucleic acid" (DNA) refers to the genetic
material while "ribonucleic acid" (RNA) is involved in
the translation of the information from DNA into
5   proteins.

The terms "genomic DNA" or "target DNA" or "target
nucleic acid" will be used interchangably and refer to
nucleic acid fragments targeted for amplification or
replication and subsequent analysis by the instant
10   method for the presence of SSR regions. Sources of
genomic DNA will typically be isolated from eukaryotic
organisms. Genomic DNA is amplified via standard
replication procedures using suitable primers to
produce detectable primer extension products.
15   The term "restriction endonuclease" or "restriction
enzyme" is an enzyme that recognizes a specific
palindromic-base sequence (target site) in a double-
stranded DNA molecule, and catalyzes the cleavage of
both strands of the DNA molecule at a particular base
20   in every target site.

The term "restriction fragments" refers to the DNA
molecules produced by digestion with a restriction
endonuclease. Any given genome may be digested by a
particular restriction endonuclease into a discrete set
25   of restriction fragments. The DNA fragments that
result from restriction endonuclease cleavage may be
separated by gel electrophoresis and detected, for
example, by either fluorescence or autoradiography.

The term "restriction fragment length polymorphism
30   (RFLP)" refers to differences in the genomic DNA of two
closely related organisms which are detected based upon
differences in the pattern of restriction fragments
generated by a restriction endonuclease digestion of
genomic DNA of the organisms. For example, a genome
35   which contains a polymorphism in the target site for a

restriction endonuclease will not be cleaved at that
point by the restriction endonuclease. Or, a
nucleotide sequence variation may introduce a novel
target site where none exists in the other organism,
5   causing the DNA to be cut by the restriction enzyme at
that point. Additionally, insertions or deletions of
nucleotides occurring between two target sites for a
restriction endonuclease in the genome of one organism
will modify the distance between those target sites.
10   Thus, digestion of the two organism's DNA will produce
restriction fragments having different lengths and will
generate a different pattern upon gel electrophoresis.
      The term "ligation" refers to the enzymatic
reaction catalyzed by the enzyme T4 DNA ligase by which
15   two double-stranded DNA molecules are covalently joined
together in their sugar-phosphate backbones via
phosphodiester bonds. Ligation can occur between two
DNA molecules that each are bounded by blunt
(nonstaggered) ends, but also can occur if the two DNA
20   molecules contain single-stranded overhanging ends that
are complementary in sequence. In general, both DNA
strands of the two double helices are covalently joined
together such that at each junction the free 5' end of
one of the DNA molecules carries a 5'-phosphate group.
25   It is also possible to prevent the ligation of one of
the two strands, through chemical or enzymatic
modification (for example, removal of the 5' phosphate)
of one of the ends, in which case the covalent joining
would occur in only one of the two DNA strands.
30   The term "adaptor" will specifically refer herein
to short, largely double stranded DNA molecules
comprised of a limited number of base pairs, e.g., 10
to 30 bp. Adaptors are comprised of two synthetic
single-stranded oligonucleotides having nucleotide
35   sequences that are not intentionally represented by

repetitive sequences in the genome of interest, and also are in part complementary to each other. Under appropriate annealing conditions, the two complementary synthetic oligonucleotides will form a partially

5   double-stranded structure in solution. At least one of the ends of the adaptor molecule is designed so that it is complementary to and can be specifically ligated to the digested end of a restriction fragment.

The term "polymerase chain reaction" or "PCR"

10   refers to the enzymatic reaction in which copies of DNA fragments are synthesized from a substrate DNA in vitro (U.S. Pat. Nos. 4,683,202 and 4,683,195). The reaction involves the use of one or more oligonucleotide primers, each of which is complementary to nucleotide

15   sequences flanking a target segment in the substrate DNA. A thermostable DNA polymerase catalyzes the incorporation of nucleotides into the newly synthesized DNA molecules which serve as templates for continuing rounds of amplification.

20   The term "DNA amplification" or "nucleic acid amplification" or "nucleic acid replication" or "primer extension" refers to any method known in the art that results in the linear or exponential replication of nucleic acid molecules that are copies of a substrate

25   DNA molecule.

The term "primer" refers to a DNA segment that serves as the initiation point or site for the replication of DNA strands. Primers generally will be single-stranded and will be complementary to at least

30   one strand of the target or substrate nucleic acid and will serve to direct nucleotide polymerization or primer extension using the targeted sequence as a template. Primers may be used in combination with another primer to "flank" the target sequence in PCR,

35   thus forming a "primer set" or "primer pair". In

general, primers are 14 to 40 nucleotides long and preferably are designed so as not to form secondary structure or hairpin configurations. Specific requirements for primer size, base sequence,

5   complementarity and target interaction are discussed in the primer section of the detailed description of the invention. The term "primer", as such, is used generally herein by Applicants to encompass any synthetic or naturally occurring oligonucleotide that

10   can hydrogen-bond specifically to a region of a substrate DNA molecule and functions to initiate the nucleic acid replication or primer extension process; such processes may include, for example, PCR, or other enzymatic reactions that employ single rather than

15   multiple oligonucleotide initiators.

The term "anchor" or anchor region" or "anchor portion" refers to a 3-20 nucleotide region of a primer designed to hybridize with a DNA sequence which is immediately adjacent to a specified sequence SSR. The

20   anchor region of a primer may occur at either the extreme 5' or 3' end, and serves to affix the primer onto the target DNA at an adjacent position relative to a specified SSR. This anchoring results in primer extension occurring from a fixed nucleotide at each

25   target site. The anchor sequence can be a nondegenerate sequence of either deliberate or arbitrary design, or it can be a fully or partially degenerate sequence. The latter would be capable of annealing to the genomic DNA sequences flanking a wide

30   range of SSR sites in a genome. Optionally, the anchor portion of the primer may be 5' end-labeled with a reporter molecule, typically a radioisotope, a fluorescent moiety or a reactive ligand.

The use of the term "arbitrary" when speaking of

35   an individual nucleotide at each position in a DNA

sequence refers to selection based on or determined by unbiased means or seemingly by chance rather than by necessity or by adherence to a predetermined sequence.

The term "non-degenerate" refers to the occurrence

5   of a single, specified nucleotide type at a particular position or at multiple specified positions in the linear ordering of nucleotides in a DNA polymer, usually an oligonucleotide or a polynucleotide. Any nondegenerate nucleotide position can carry an intended

10 base (either A, G, C or T) that is known for example to correspond to a given template site, or it can carry an arbitrarily chosen base, which will correspond to a target site that is not known *a priori*. A "non-degenerate oligonucleotide" means that every nucleotide

15 position within the DNA molecule is non-degenerate. The term "degenerate" refers to the occurrence of more than one specified nucleotide type at a particular position in an oligonucleotide or polynucleotide. A specific oligonucleotide can be made up of some

20 positions that are degenerate and some positions that are fully or partially degenerate. "Fully degenerate" indicates the presence of an equal mixture of the four possible nucleotide bases (A, G, C or T) at a particular nucleotide position; partially degenerate

25 indicates the presence of only two or three of the four possible bases at a particular position. A "degenerate oligonucleotide" is one in which at least one position within it carries full or partial degeneracy; such an oligo- or polynucleotide is a mixture of specific,

30 nondegenerate DNA molecules, each of which represents a single permutation of the nucleotide sequences possible by virtue of the degenerate base(s) specified in the linear nucleotide sequence. An oligonucleotide with two fully degenerate positions, for example, would be a

35 mixture of $(4)^2 = 16$ different nondegenerate molecules;

an oligonucleotide with four fully degenerate and two
partially degenerate (three bases) positions would
comprise a mixture of $(4)^3 \times (3)^2 = 576$ different non-
degenerate molecules. Standard degeneracy codes used
5   herein are:

N or X	A, G, C or T
B	G, C or T [anything except A]
D	A, G or T [anything except C]
H	A, C or T [anything except G]
V	A, C or G [anything except T]

The term "reporter" or "reporter molecule" refers
to any moiety capable of being detected via enzymatic
means, immunological means or energy emission;
15   including, but not limited to, fluorescent molecules,
radioactive tags, light emitting moieties or
immunoreactive or affinity reactive ligands.

The term "binding pair" includes any of the class
of specific inter-molecular or recognition immune-type
20   binding pairs, such as antigen/antibody or hapten/anti-
hapten systems; and also any of the class of nonimmune-
type binding pairs, such as biotin/avidin;
biotin/streptavidin; folic acid/folate binding protein;
complementary nucleic acid segments; protein A or
25   G/immunoglobulins; and binding pairs which form
covalent bonds, such as sulfhydryl reactive groups
including maleimides and haloacetyl derivatives, and
amine reactive groups such as isothiocyanates,
succinimidyl esters and sulfonyl halides.

30   The present invention describes the design and use
of self-anchoring primers for the detection of SSR
genetic markers. The general polymorphism detection
method using these primers is termed selective
amplification of microsatellite polymorphic loci
35   (SAMPL). The method of primer design is based on the
observation that many compound SSR sequences are

composed of dinucleotide repeats wherein a single type
of nucleotide is shared by both of the directly
adjacent constituent repeats and is maintained "in-
phase" across the repeat junction and throughout the
5   length of the repeat. The present invention combines
many of the advantages inherent to conventional,
single-locus SSR markers (i.e., high levels of
polymorphs and high codominance potential), with the
added benefits and convenience offered by multiplexed
10 genome assays. As with conventional single-locus SSR
markers, the use of perfect compound SSR sequences as
self-anchored PCR primers enables the identification of
dominant and codominant polymorphisms between genomes.
However, unlike conventional SSR analysis, this method
15 requires no prior knowledge of the unique sequences
flanking individual SSR loci. Thus, no labor-intensive
SSR marker discovery or locus identification is
necessary to use such compound SSR sequences as primers
as we describe. Also, as with conventional SSR
20 markers, the in-phase subset of compound SSR sequences
appears to be highly abundant and well dispersed in
both plant and animal genomes, as well as to be highly
polymorphic between individual genomes. In contrast,
the "out-of-phase" subset of perfect compound SSR
25 sequences are represented in these same genomes at much
lower relative frequencies. Therefore, the likelihood
that the highly abundant, in-phase perfect compound SSR
sequences can identify new polymorphisms closely linked
to loci of interest is extremely high. Additionally,
30 PCR primers representing these compound SSR sequences
are self-anchoring, such that the 5'-most repeat serves
as the anchor for primer extension by the 3'-most of
the two repeats, thus obviating the need to incorporate
into these primers any additional degenerate or fixed
35 sequences as 5' or 3' flanking anchors. Therefore,

conceivably every genomic locus harboring the same
compound SSR sequence would be expected to serve as a
target site for simultaneous amplification by the
single compound SSR primer matching these target sites.

5   Finally, and most preferred, the use of these primers
can be incorporated into several different genome
assays to increase their versatility and informativity.
For example, the use of these perfect, in-phase
compound SSR primers in modifications of the amplified

10   fragment length polymorphism assay (AFLP; Zabeau,
EP 534,858) leads to an increase in the proportion of
amplification products that are polymorphic and
codominant between even highly related genomes as
compared to conventional AFLP methods. The versatility

15   of these compound SSR primers, in combination with the
ability to fine-tune both the numbers and types of
amplification products achievable with the AFLP assay,
offers a unique combination of benefits for the
multiplexed analysis of complex plant and animal

20   genomes.

Applicants' modified AFLP invention is illustrated
in Figure 1. Genomic DNA (I) carrying perfect in-phase
compound SSR sequences at some frequency of occurrence
is digested with a single restriction enzyme, or with a

25   combination of two or more restriction enzymes. This
Figure demonstrates the use of a double enzyme
combination, one enzyme having a hexanucleotide
recognition site (hatched boxes) and the other a
tetranucleotide site (dark boxes). It is also within

30   the scope of the invention to choose other combinations
of multiple restriction enzymes, including but not
limited to the following combinations of restriction
enzymes with the specified types (lengths) of
recognition site:

35

Additional Enzyme combinations

two enzymes	three enzymes
4 + 4	4 + 4 + 4
5 + 5	5 + 4 + 4
6 + 6	6 + 4 + 4
4 + 5	5 + 4 + 5
5 + 6	6 + 4 + 5
4 + 8	6 + 4 + 6
5 + 8	5 + 5 + 5
	5 + 5 + 6
	6 + 6 + 6

In all cases, these multiple enzyme digestions produce
a mixture of restriction fragments with all
combinations of the corresponding blunt or single
5   stranded overhanging ends. Additionally it is within
the scope of the invention for a single restriction
enzyme to be used to produce fragments all sharing the
same blunt or single-stranded overhanging ends. In
general, any enzyme with a 4-, 5-, 6- or 8-bp
10   recognition site is suitable, providing the enzyme's
activity is not affected or inhibited by DNA
methylation or other, nonmendelian modes of DNA
modification within the enzyme site.

Next, double stranded adaptors A and B are
15   constructed wherein Adaptor A anneals specifically to
the single stranded overhang produced by the
hexanucleotide-site enzyme, and Adaptor B to the
overhang left by the tetranucleotide-site enzyme.
Adaptors A and B are simultaneously ligated to the
20   appropriate ends of all the restriction fragments (II)
in the digested DNA mixture using standard methods
(also described in Table III). In an alternate
embodiment, either Adaptor A or Adaptor B may be
conjugated with a member of a binding pair such as

biotin (as part of a biotin-streptavidin pair),
allowing capture and isolation of a smaller subset of
the genomic restriction fragments (III). Either of the
adaptors can be so modified; the proportion of genomic
5   fragments then selected for is affected by the genomic
fequency of the specific restriction site recognized by
the modified adaptor. For any given restriction
enzyme combination, this enrichment method
categorically allows only a small fraction of the total
10   number of restriction fragments from a genome the
opportunity to serve as template for the subsequent PCR
amplifiction; however, this reduced complexity is
necessary for ensuring a manageable number of co-
amplified products in the next step. The entire genome
15   can be examined through the use of multiple
combinations of restriction enzymes for the generation
of different sets of enriched genomic fragments.
Figure 1a illustrates a biotinylated hexanucleotide
site adaptor.
20   Alternatively, the complexity of the genomic
fragment mixture can be selectively reduced by
performing a pre-amplification step prior to the final
amplification, using a pair of unlabeled adaptor-
directed primers. This primer pair comprises two
25   different primers, one corresponding to one adaptor
sequence and the second primer to the other adaptor
sequence. Each primer carries one selective nucleotide
at its 3'-end. Since the 3'-most position of each of
these +1 adaptor primers can be occupied by any one of
30   the four DNA nucleotides, each adaptor can be
represented by 4 different primers. Furthermore,
4x4=16 different combinations of these +1 primers can
be used against any genomic fragment mixture to
generate 16 different, nonoverlapping fragment subsets
35   from each genome. Thus, the pre-amplification enriches

for a subset of the total mixture of genomic fragments, and different enriched subsets can be generated from a single restriction fragment mixture by varying the specific primers used for the pre-amplification.

5   No matter how the fragment enrichment is performed, a pair of PCR primers next are synthesized according to standard protocols. One primer will be an adaptor-directed primer, designed to anneal to adaptor B specifically when the biotin-mediated fragment

10 enrichment method is employed. For enrichment via pre-amplification, this primer can correspond to either of the two adaptors. In either case, this adaptor primer carries 1-4 randomly selected nucleotides at their 3'-ends.

15   The other primer will be a SSR-directed primer, designed to anneal specifically to a particular SSR sequence represented on a subset of the genomic fragments. In a preferred embodiment, the SSR-directed primer is 5'-end labeled, typically with a

20 radionucleotide such as ^{32}P or ^{33}P or with a fluorescent moiety (*). It is further especially preferred if the SSR-directed primer is of the "perfect compound" type wherein the primer straddles the compound SSR, preferably with one nucleotide remaining

25 "in-phase" across the length of the primer. Exponential amplification of a subset of the adaptor modified restriction fragments in the presence of the adaptor-directed primer and the 5'-labeled SSR primer generates labeled primer extension products (IV) from

30 every input genomic fragment that carries the SSR sequence and is bordered at the opposing end by the designated adaptor sequence.

    This method generates multiple co-amplification products, a high proportion of which are expected to be

35 polymorphic between genomes. Those genomic fragments

lacking either the designated SSR sequence or the appropriate adaptor end, or both, will not be exponentially amplified, and therefore will not be detected (V).

5   GENERAL METHODS:

Primer Design:

All oligonucleotides primers are synthesized using solid-phase phosphoramidite chemistry such as that described by Operon Technologies, Alameda, CA. All

10   primers are non-phosphorylated at their 5'ends and can be used in unpurified form providing efficient syntheses. However, oligonucleotides purified by column chromatography are preferred for optimal primer specificity. All oligonucleotide primer sequences are

15   chosen such that the T_m in 50 mM salt (KCl or NaCl) is between 38° and 45°C (as determined using the algorithm employed by Oligo v4.03 for the Macintosh, National Biosciences, Inc.).

Several types of primers are used within the

20   context of Applicants' invention. The first is an adaptor-directed primer (as disclosed in Zabeau EP 534,858) which can vary from 15 to 25 nucleotides in length, and from 40% and 60% G+C. Starting at its 5' end, the primer spans the length of, and is

25   complementary to, one strand of a double-stranded adaptor that is ligated to the restriction endonuclease-digested target DNA to be tested. The primer then covers all or part of the restriction site, and its 3' end can carry arbitrary, nondegenerate bases

30   that anneal to and prime from nucleotides within the target DNA fragment adjacent to the adaptor. The sequence of such an adaptor-directed primer can vary, depending upon the specific adaptor used for the construction of the template and upon the number of

35   arbitrary, selective nucleotides positioned at its

3'-end. Examples of DNA sequences and characteristics
of oligonucleotides comprising both adaptor and
adaptor-directed primers, each specific to the site
generated by a particular restriction enzyme, are given
5   in but not limited to Table I.

TABLE I

ADAPTOR AND PRIMER OLIGONUCLEOTIDES

Double Strand Adaptor	Oligonucleotide Name	Sequence(5'→3')/SEQ ID No.	Length	Selective 3' nts	Tm (50mM Salt)	%GC
Adaptor oligonucleotide components						
biotin-Pst I-Ad	B-Pst.Ad.F	B-CTCGTAGACTGCGTACATGCA /17	21	0	49.2°C	52.40
	Pst.Ad.R	3'-CATCTGACGCATGT-5' /18	14	0	25.7°C	50.00
biotin-Hnd III-Ad	B-Hnd.Ad.F	B-CTCGTAGACTGCGTACC /19	17	0	44.3°C	58.80
	Hnd.Ad.R	3'-CTGACGCATGGTCGA-5' /20	15	0	39.5°C	60.00
Taq I-Ad	TAq.Ad.F	GACGATGAGTCCTGAC /21	16	0	40.8°C	56.20
	TAq.Ad.R	3'-TACTCAGGACTGGC-5' /22	14	0	35.1°C	57.10
Sau-Ad	Sau.Ad.F	GGAATTCTGGACTCAGT /23	17	0	39.5°C	47.00
	Sau.Ad.R	3'-CCTTAAGACCTGAGTCACTAG-5' /24	21	0	47.3°C	47.60
Mse-Ad	Mse.Ad.F	TGGCCTTTACAGCGTC /25	16	0	40.8°C	56.30
	Mse.Ad.R	3'-GAAATGTCGCACAT-5' /26	14	0	29.3°C	42.90
Adaptor-directed primers						
	Hnd.Ad.F	B-CTCGTAGACTGCGTACC /27	17	0	44.3°C	58.80
	Hnd.pr.1	CTGCGTACCAGCTTaca /28	17	3 -aca	41.9°C	52.90
	Hnd.pr.2	CTGCGTACCAGCTTacc /29	17	3 -acc	44.3°C	58.80
	Hnd.pr.3	CTGCGTACCAGCTTaac /30	17	3 -aac	41.9°C	52.90
	Hnd.pr.4	CTGCGTACCAGCTTgtc /31	17	3 -gtc	44.3°C	58.80
	Hnd.pr.5	CTGCGTACCAGCTTac /32	16	2 -ac	40.8°C	56.20
	Hnd.pr.6	CTGCGTACCAGCTTaa /33	16	2 -aa	38.2°C	47.05

Name	Sequence					
Pst.Ad.F	B-CTCGTAGACTGCGTACATGCA /34	21	0		49.2°C	52.40
Pst.pr.1	GACTGCGTACATGCAGac /35	18	2	-ac	45.2°C	55.60
Pst.pr.2	GACTGCGTACATGCAGaa /36	18	2	-aa	42.9°C	50.00
Pst.pr.3	GACTGCGTACATGCAGca /37	18	2	-ca	45.2°C	55.60
Pst.pr.4	GACTGCGTACATGCAGtt /38	18	2	-tt	42.9°C	50.00
Pst.pr6	GACTGCGTACATGCAGa /39	17	1	-a	41.9°C	52.94
Pst.pr8	GACTGCGTACATGCAGc /40	17	1	-c	44.3°C	58.82
Taq.Ad.F	GACGATGAGTCCTGAC /41	16	0		40.8°C	56.20
Taq.pr.0	ATGAGTCCTGACCGA /42	15	0		36.8°C	53.30
Taq.pr.1	TGAGTCCTGACCGAacc /43	17	3	-acc	44.3°C	58.80
Taq.pr.2	TGAGTCCTGACCGAaca /44	17	3	-aca	41.9°C	52.90
Taq.pr.3	TGAGTCCTGACCGAcac /45	17	3	-cac	44.3°C	58.80
Taq.pr.4	TGAGTCCTGACCGAcaa /46	17	3	-caa	41.9°C	52.90
Taq.pr.5	ATGAGTCCTGACCGAg /47	16	1	-g	39.5°C	60.00
Taq.pr.6	ATGAGTCCTGACCGAa /48	16	1	-a	36.8°C	53.30
Taq.pr.7	ATGAGTCCTGACCGAt /49	16	1	-t	36.8°C	53.30
Taq.pr.8	ATGAGTCCTGACCGAc /50	16	1	-c	39.5°C	60.00
Taq.pr.9	TGAGTCCTGACCGAac /51	16	2	-ac	40.8°C	56.20
Taq.pr.10	TGAGTCCTGACCGAaa /52	16	2	-aa	38.2°C	50.00
Taq.pr.11	TGAGTCCTGACCGAca /53	16	2	-ca	40.8°C	56.20
Sau.Ad.F	GGAATTCTGGACTCAGT /54	17	0		39.5°C	47.10
Sau.pr.0	GGAATTCTGGACTCAGTGATC /55	21	0		47.3°C	47.70
Sau.pr.1T	TTCTGGACTCAGTGATCt /56	18	1	-t	40.6°C	44.40
Sau.pr.2TT	TCTGGACTCAGTGATCtt /57	18	2	-tt	40.6°C	44.40
Sau.pr.3TTC	CTGGACTCAGTGATCttc /58	18	3	-ttc	42.9°C	50.00
Mse.Ad.F	TGGCCTTACAGCCGTC /59	16	0		40.8°C	56.30
Mse.pr.1T	GCCTTACAGCGTCTAat /60	18	1	-t	40.6°C	44.40
Mse.pr.2TC	CCTTACAGCGTCTAatc /61	18	2	-tc	40.6°C	44.40
Mse.pr.3TCA	CCTTACAGCGTCTAAtca /62	19	3	-tca	41.6°C	42.10

A second type of primer used within the present
invention corresponds in its 3' portion to a simple
sequence repeat, or microsatellite, where the structure
of the microsatellite is simple (simple SSR) as defined
5   above. The simple microsatellite region can be tandem
repeats of mono-, di-, tri-, tetra- or penta-
nucleotides. The 5' position of the primer contains 3
to 5 fully or partially degenerate nucleotides, which
serve to anchor the primer adjacent to a microsatellite
10 in the targeted genome. This primer can vary in length
from 10 to 60 nucleotides, with a [G+C] content
typically from 16% to 80%. Length polymorphism within
a microsatellite locus between genomes is expected to
be detectable using these primers, since in nearly all
15 cases, primer extension is expected to initiate from a
fixed site relative to the SSR target region. Primers
similar to these are described by Zietkiewicz, E., et
al., *Genomics*, 20, 176, (1994).
        Another type of SSR-directed primer, uniquely
20 utilized within the present invention, is the perfect
compound SSR primer which is comprised of two different
perfect SSR's that are immediately adjacent to one
another with no intervening nucleotides either between
the repeats or within each of the repeats. Perfect
25 compound SSR primers are perfectly self-anchoring; that
is, the simple SSR at the 5'-end of the primer serves
as an efficient anchor for the adjacent 3' SSR from
which primer extension proceeds (see Figure 1c). It is
intended, therefore, that primer extension initiates
30 from a single, fixed site within a compound SSR target
region. Any length variation between genomes in the
portion of the target SSR across which primer extension
occurs should be visible as length variation in the
resulting amplification products from those genomes.
35 The relative lengths of each constituent SSR within the

compound primer can vary. However, Applicants have
found that the best primer anchoring and greatest
specificity for the target template is produced when
the length of the 5' anchor is equal to or greater than
5   that of the 3' priming portion.

For dinucleotide repeats, Applicants theorize
that, excluding CG and GC combinations (long stretches
of which are thought to be rare in eukaryotic genomes),
90 different permutations of two adjacent dinucleotide
10   sequences are possible. As estimated by the following
equation,

$$[(4)(3)-2] \times [[(4)(3)-2-1] = 90,$$

the first (5'-most) constituent repeat may carry of any
of the four nucleotides (A, G, C or T) in its first
15   position, followed by any of the three remaining
nucleotides at its second position, then from this
product should be subtracted the two GC and CG
combinations. For the second (3'-most) dinucleotide,
the same calculation holds, but with the additional
20   subtraction of the one combination occupying the first
constituent dinucleotide repeat position. All of these
90 permutations are listed in Table II. Only 80 are
true compound repeat sequences, however; 10 of the
permutations are actually imperfect simple repeats
25   (e.g., $(CT)_n(TC)_n$, $(AG)_n(GA)_n$, etc.). Similar
calculations can be performed to estimate the number
and types of different tri-, tetra- and penta-
nucleotide combinations possible by random nucleotide
arrangements.

TABLE II

Permutations of Adjacent Dinucleotides:

COMPOUND, IN-PHASE Double Stranded Locus	Permutations 5'→3'	Total, all databases	Primate + human EST	Occurrence in DNA Sequence Database									
				Rodent	Other Mammal	Other Vertebrate	Invertebrate	Bacteria	Virus	Phage	Fungi	Plant	Cloned Soybean
5'-(AC)x(AT)y-3' 3'-x(TG)y(TA)-5'	(AC)(AT),(CA)(TA) (AT)(GT),(TA)(TG)	79	28	27	5	2	8	0	0	0	5	4	22
5'-(AT)x(AC)y-3' 3'-x(TA)y(TG)-5'	(TA)(CA),(AT)(AC) (TG)(TA),(GT)(AT)	65	47	6	7	3	1	0	0	0	0	1	4
5'-(AG)x(AT)y-3' 3'-x(TC)y(TA)-5'	(AG)(AT),(GA)(TA) (AT)(CT),(TA)(TC)	0	0	0	0	0	0	0	0	0	0	0	1
5'-(AT)x(AG)y-3' 3'-x(TA)y(TC)-5'	(TA)(GA),(AT)(AG) (TC)(TA),(CT)(AT)	15	5	0	3	1	1	0	0	0	0	5	11
5'-(AC)x(AG)y-3' 3'-x(TG)y(TC)-5'	(AC)(AG),(CA)(GA) (CT)(GT),(TC)(TG)	83	40	41	2	0	0	0	0	0	0	0	3
5'-(AG)x(AC)y-3' 3'-x(TC)y(TG)-5'	(AG)(AC),(GA)(CA) (GT)(CT),(TG)(TC)	3	2	1	0	0	0	0	0	0	0	0	2 (39)
5'-(TG)x(AG)y-3' 3'-x(AC)y(TC)-5'	(TG)(AG),(GT)(GA) (CT)(CA),(CA)(CT)	221	98	43	71	2	4	0	2	0	0	1	5
5'-(AG)x(TG)y-3' 3'-x(TC)y(AC)-5'	(AG)(TG),(GA)(GT) (CA)(CT),(AC)(TC)	65	56	5	1	0	3	0	0	0	0	0	1
total occurrences # bp searched		531 2.0E+08	276 3.3E+07	123 2.5E+07	89 6.7E+06	8 8.0E+06	17 2.2E+07	0 3.0E+07	2 2.2E+07	0 1.5E+06	5 3.1E+06	11 >>	49
# sequences searched		2.0E+05	3.5E+04	2.2E+04	6.2E+03	7.4E+03	1.3E+04	1.7E+04	1.8E+04	9.9E+02	1.8E+04	>>	

COMPOUND, OUT-OF-PHASE Double Stranded Locus	Permutations 5' → 3'	Total, all databases
5'-(AC)x(TA)y-3' 3'-x(TG)y(AT)-5'	(AC)(TA) (TA)(GT)	1
5'-(AC)x(GA)y-3' 3'-x(TG)y(CT)-5'	(AC)(GA) (TC)(GT)	7
5'-(AT)x(CA)y-3' 3'-x(TA)y(GT)-5'	(AT)(CA) (TG)(AT)	2
5'-(AT)x(GA)y-3' 3'-x(TA)y(CT)-5'	(AT)(GA) (TC)(AT)	2
5'-(AG)x(CA)y-3' 3'-x(TC)y(GT)-5'	(AG)(CA) (TG)(CT)	0
5'-(AG)x(TA)y-3' 3'-x(TC)y(AT)-5'	(AG)(TA) (TA)(CT)	0
5'-(CA)x(TC)y-3' 3'-x(GT)y(AG)-5'	(CA)(TC) (GA)(TG)	0
5'-(CT)x(AC)y-3' 3'-x(GA)y(TG)-5'	(CT)(AC) (GT)(AG)	7
5'-(AG)x(GT)y-3' 3'-x(TC)y(CA)-5'	(AG)(GT) (AC)(CT)	2
5'-(TG)x(GA)y-3' 3'-x(AC)y(CT)-5'	(TG)(GA) (TC)(CA)	3
5'-(GT)x(TA)y-3' 3'-x(CA)y(AT)-5'	(GT)(TA) (TA)(AC)	0
5'-(AT)x(TG)y-3' 3'-x(TA)y(AC)-5'	(AT)(TG) (CA)(AT)	1

Note: x, y ≥6 for all dinucleotide combination searches

double stranded locus	permutations 5'->3'	
5'-(CT)x(TA)y-3' 3'-x(GA)y(AT)-5'	(CT)(TA) (TA)(AG)	0
5'-(AT)x(TC)y-3' 3'-x(TA)y(AG)-5'	(AT)(TC) (GA)(AT)	0
5'-(GT)x(TC)y-3' 3'-x(CA)y(AG)-5'	(GT)(TC) (GA)(AC)	1
5'-(CT)x(TG)y-3' 3'-x(GA)y(AC)-5'	(CT)(TG) (CA)(AG)	0
	total occurrences	26

COMPOUND, NONPALINDROMIC, NO SHARED NTS BETWEEN REPEATS

double stranded locus	permutations 5'->3'	
5'-(GA)x(CT)y-3' 3'-x(CT)y(AG)-5'	(GA)(CT) (AG)(TC)	0
5'-(CT)x(GA)y-3' 3'-x(GA)y(CT)-5'	(CT)(GA) (TC)(GA)	0
5'-(CA)x(GT)y-3' 3'-x(GT)y(CA)-5'	(CA)(GT) (AC)(TG)	0
5'-(GT)x(CA)y-3' 3'-x(CA)y(GT)-5'	(GT)(CA) (TG)(AC)	2
	total occurrences	2

COMPOUND, PALINDROMIC, NO SHARED NTS BETWEEN REPEATS

double stranded locus	permutations 5'->3'	
5'-(GA)x(TC)y-3'	(GA)(TC)	0
5'-(AG)x(CT)y-3'	(AG)(CT)	0
5'-(TC)x(GA)y-3'	(TC)(GA)	0
5'-(CT)x(AG)y-3'	(CT)(AG)	0
5'-(CA)x(TG)y-3'	(CA)(TG)	1
5'-(AC)x(GT)y-3'	(AC)(GT)	0
5'-(TG)x(CA)y-3'	(TG)(CA)	1
5'-(GT)x(AC)y-3'	(GT)(AC)	2
	total occurrences	2

NOT COMPOUND; IMPERFECT SIMPLE REPEATS

double stranded locus	permutations 5'->3'	
5'-(CA)x(AC)y-3'	(CA)(AC)	13
3'-x(GT)y(TG)-5'	(GT)(TG)	18
5'-(AC)x(CA)y-3'	(AC)(CA)	
3'-x(TG)y(GT)-5'	(TG)(TG)	4
5'-(GA)x(AG)y-3'	(GA)(AG)	
3'-x(CT)y(TC)-5'	(CT)(TC)	9
5'-(AG)x(GA)y-3'	(AG)(GA)	
3'-x(TC)y(CT)-5'	(TC)(CT)	20
5'-(AT)x(TA)y-3'	(AT)(TA)	
3'-x(TA)y(AT)-5'	(TA)(AT)	
	total occurrences	64

The complete GenBank DNA sequence databases
(version 84.0) were searched using the FindPatterns
search algorithm within the University of Wisconsin
Genetics Computer Group sequence analysis package

5   (version 7.3). The individual strands of the double
stranded sequences shown in the first column were used
individually as queries either against the entire
GenBank database (all species combined) or against the
separate subdatabases representing the indicated

10   phylogenetic groupings. For each query, such as
$(AC)_x(AT)_y$, x and y were each designated to be ≥ 6; that
is, any hit in the database was required to carry at
least 6 units of each of the two constituent
dinucleotide repeats. The final column designates the

15   number of matches to the respective query within an
in-lab collection of cloned soybean SSR sequences
(unpublished data), isolated as small inserts
containing either $(AC)_x$ or $(AG)_y$ sequences.

Although all 80 compound dinucleotide sequences

20   have the potential to exist in a genome and to serve as
targets for corresponding primers, only a small subset
of these adjacent dinucleotide repeat combinations has
been observed to occur at a reasonable frequency among
compound SSRs represented within the DNA sequence

25   databases and among SSRs cloned and sequenced from
plant and animal genomes (see Table II). The great
majority of these 80 total combinations occur at
surprisingly low relative frequencies in eukaryotic
genomes.

30   Nearly all of the compound repeats in this high
frequency subset have perfect nucleotide periodicity
whereby the two adjacent constituent dinucleotide
repeats share a common nucleotide that retains a
constant periodic spacing across the junction and the

35   entire compound structure. These compound SSR

sequences are designated by Applicants as "in-phase", and include repeats such as $(AT)_x(AG)_y$, $(AC)_x(TC)_y$, and $(AT)_x(GT)_y$ where x and y independently are ≥ 2 and can be multiples of 0.5. Thirty-two of the 80 possible
5   dinucleotide combinations fall into this in-phase category, and these are listed in Table II. All the remaining compound repeats are termed, "out-of-phase". The 32 individual in-phase compound sequences (excluding CG and GC), however, represent only 16
10   unique nonredundant single-stranded sequences. The 2-fold redundancy derives from the possibility of positioning the repeating in-phase nucleotide as either the first or the second base in the core repeats (i.e., $(AC)_x(AT)_y$ and $(CA)_x(TA)_y$, although nonidentical as
15   individual primers, represent the same compound sequence). In addition, these 16 canonical, nonredundant sequences represent the complementary strands of only 8 individual double-stranded compound repeat loci (see Table II). In other words, a compound
20   dinucleotide repeat as a locus in double stranded DNA could be recognized by any of four different single stranded oligonucleotide primers, out of the total set of 32 possible permutations. For example, a locus $(AT)_6(AG)_6$, is a target site for the four hypothetical
25   primers, $(AT)_x(AG)_y$ $(TA)_x(GA)_y$ $(CT)_x(AT)_y$, and $(TC)_x(TA)_y$ (with x, y ≤ 6).

By chance alone, and assuming no nucleotide bias in a source genome, each of the 80 different perfect compound dinucleotide permutations would be expected to
30   occur in the genome at equal frequencies. As mentioned above, however, it was already discovered by Applicants that the in-phase subset are more abundant compared to the out-of-phase set. Further, the two possible permutations of the constituent repeats for some of the
35   in-phase compound sequence combinations appear to be

represented at widely differing frequencies in a given genome. For example, the compound repeat, $(AT)\leq_6$ $(AG)\leq_6$ appears to be at least 5 times more abundant in plant genomes than its permutant counterpart,

5   $(AG)\leq_6$ $(AT)\leq_6$; and, $(AC)\leq_6$ $(AG)\leq_6$ is much more abundant in primate genomes than $(AG)\leq_6$ $(AC)\leq_6$. Both from a systematic analysis of cloned sequence databases and from an empirical examination of both plant and animal genomes, these few, most frequently occurring compound

10 dinucleotide repeats are known by Applicants and therefore are fully predictable. This knowledge serves to reduce to only a few the number of different compound SSR primers that will be successful for producing an adequate number of SSR-to-adaptor co-

15 amplification products using the present invention.

Thus, of the 80 total compound dinucleotide sequence permutations that are possible by random arrangement of nucleotides, only a few (nearly all of which are in-phase) are present in plant and animal

20 genomes at a measurable frequency, and only these few, therefore, are required to detect a large proportion of the compound SSR loci present in any given genome. Experimental data demonstrate, however, that specific primers representing these 16 compound sequences are

25 not equally effective at recognizing and priming from the respective target locus. Such differences in primer efficiency were determined empirically to result from each constituent repeat's base composition (AT-richness), in combination with the relative

30 position (5' anchoring versus 3'-priming) of each constituent repeat within the primer.

Table II also lists the dinucleotide permutations for which the spacing of a shared nucleotide is not preserved (termed, "out-of-phase") or for which no

35 nucleotide is shared between the two constituent

repeats. The latter category contains dinucleotide combinations that are both palindromic and nonpalindromic. Although not nearly as frequent in eukaryotic genomes as the in-phase sequences, some of
5 the out-of-phase compound SSR sequences nonetheless appear to be present in most genomes. Therefore, primers that correspond to such out-of-phase repeats also are expected to serve as initiation sites for primer-extension from their respective target loci in
10 the genome. Preferred anchored primers of the instant invention where primer nucleotide periodicity is not specifically designated may be defined by formula I for dinucleotide repeats:

Formula I

15 5'- $(XY)_{\leq 15}$ $(NM)_{\leq 15}$ -3'

where X = A, C, T, or G ; Y = A, C, T, or G ; X_Y
N = A, C, T, or G ; M = A, C, T, or G ; N_M
and where XY ≠ NM

Herein, these are abbreviated as (XY)#(NM)#
20 and by formula II for trinucleotide repeats:

Formula II

5'-$(XYZ)_{\leq 10}$ $(LMP)_{\leq 10}$ -3'

where X = A,C,T,or G; Y = A,C,T,or G; Z=A,C,T,or G
and X, Y, Z are not the same single base;
25 where M = A,C,T,or G; N = A,C,T,or G; P=A,C,T,or G
and M, N, P are not the same single base;
and where XYZ ≠ NMP

Generally the primers of formulae I and II will consist of oligonucleotides of 10-60 nucleotides in
30 length that contain two different, constituent simple sequence repeats that are directly adjacent to one another, with no intervening non-repeat nucleotides. From the 5' end, this oligonucleotide contains a simple sequence repeat of up to 15 repeat units in length,

followed immediately 3' by a second simple sequence
repeat that is also up to 15 repeat units in length.

Preferred anchored primers of the instant
invention where primer nucleotides are specifically
5   designated to be in-phase may be defined by the
formula III for dinucleotide repeats:

Formula III:

This formula describes a subset of sequences
covered broadly by Formula I.

10   5'- $(XY)\leq_{15}$ $(XZ)\leq_{15}$ -3'   or   5'- $(YX)\leq_{15}$ $(ZX)\leq_{15}$ -3'
where X = A,C,T,or G; Y = A,C,T,or G; Z =A,
C,T,or G

but where $Y \neq X$; $Z \neq X$; and $Y \neq Z$

Herein, these are abbreviated as (XY)#(XZ)# or
15   (YX)#(ZX)#.

Typically oligonucleotides of formula III are
10-60 nucleotides in length and contain two different,
constituent simple sequence repeats that are directly
adjacent to one another, with no intervening nonrepeat
20   nucleotides. From the 5' end, this oligonucleotide
contains a simple sequence repeat of up to 15 repeat
units in length, followed immediately 3' by a second
simple sequence repeat that is also up to 15 repeats.
Each repeat shares a common nucleotide, which is
25   retained at a consistent periodicity across both
constituent repeats, occupying either the first or the
second position within each repeat.

In an especially preferred embodiment of
Applicants' invention, PCR amplification to detect
30   polymorphisms is carried out using restriction
fragmented DNA modified with appropriate adaptors,
wherein the primer pair used is comprised of one primer
which is of the first type described above (Zabeau
EP 534,858; an AFLP primer) and the second primer is

one of Applicants' unique perfect compound SSR primers described above.

One of skill in the art will also appreciate that Applicants' unique compound SSR primers can also be
5 used in conjunction with a variety of other primer types which include for example, non-adaptor primers, primers of fixed sequence, arbitrary primers or any primer that might hybridize with currently known or unknown dispersed repeated sequences in the genome. An
10 example particularly suited to the present invention would be where the other primer is of completely arbitrary sequence such as a RAPD primer (Williams et al., Nucleic Acids Res. 18, 6531, (1990)).

RAPD primers have been used to generate
15 polymorphic markers from the amplification of genomic DNA. Preferably the nucleotide sequence of the RAPD primers would be about 9 to 10 bases in length, between 50 and 80% G+C in composition and contain no palindromic sequences. Amplifications using RAPD
20 primers alone are typically done using short primers and low annealing temperatures which maximizes the probability that several randomly distributed loci on the genome will produce amplification products. Because the incidence of any particular RAPD binding
25 site within the genome is relatively low, this methodology would serve to restrict the amount of the genome that is subject to amplification with any particular primer combination. It is contemplated that the selectivity of the RAPD methodology would serve an
30 enrichment function, similar to the selection function provided by use of a biotinylated adaptor in the conventional AFLP method to enrich for only a subset of randomly distributed genomic regions, from which a manageable number of co-amplification could then occur.

PREPARATION OF GENOMIC DNA:

Restriction digested fragments:

Target DNA useful for amplification in the present
invention was comprised of restriction fragments
5   generated from Taq I + Pst 1 or Taq I + HindIII
digestion of eukaryotic genomic DNA, further modified
by the ligation of specific adaptor sequences. Genomic
DNA was isolated from soybean and corn using either the
CTAB/chloroform extraction and CsCl/centrifugation
10   method of Murray and Thompson (Murry et al., *Nuc. Acid
Res.*, 8, 4321, 1980) or a urea extraction miniprep
procedure (Chen et al., The Maize Handbook, M. Freeling
and V. Walbot, eds., (1993) pp 526-527, New York).
Mammalian and salmon genomic DNAs were purchased from
15   commercial sources (Sigma (St. Louis, MO); Clontech
(Palo Alto, CA)). Genomic DNA was prepared for
amplification reactions by complete restriction
endonuclease digestion followed by ligation of site-
specific double-stranded adaptors. Methods for this
20   type of adaptor design and construction are well known
in the art, and examples are given by Zabeau,
EP 534,858.

It is preferred if a combination of two different
restriction enzymes having 4-bp and 6-bp recognition
25   sites, respectively, are used for the preparation of
target DNA. Examples of suitable restriction enzymes
are Taq I and Pst I however, any restriction enzymes
having 4-, 5-, 6- or even 8-bp recognition sites also
are appropriate providing their activities are not
30   inhibited by target site DNA methylation or other non-
mendelian mechanisms of selective nucleotide
modification. Any combination of such restriction
enzymes are potentially suitable. Other restriction
enzymes suitable for the present invention may include
35   but are not limited to the hexanucleotide site enzymes,

EcoRI, DraI or BamHI, the tetranucleotide stie enzymes, Sau 3AI, MboI, MseI, Tsp509I or AluI, the pentanuleotide site enzymes HinfI or AvaII, and the octanucleotide site enzymes, PmeI, PacI, or SwaI.

5   Genomic DNA is digested with a first enzyme such as Taq I, followed by further digestion with a second enzyme such as Pst I, according to standard protocols, such as that given by Zabeau (EP 534,858). The digestions generated from each input genomic DNA are a
10   mixture of symmetric fragments, bordered at both ends by either Taq I or Pst I sites, and asymmetric fragments, each flanked by a Taq I site and a Pst I site.

Adaptors:

15   Double stranded adaptors are generated by annealing the two partially complementary single stranded component oligonucleotides of each pair (examples are listed in Table I). Since restriction endonucleases cleave genomic DNA molecules at specific
20   sites, amplification of restriction fragments can be achieved by first ligating synthetic oligonucleotide adaptors to the ends of restriction fragments, thus providing all restriction fragments with two common flanking tags which will serve as anchor bases for the
25   primers used in PCR amplification. Typically, restriction enzymes either produce flush ends on a DNA fragment, such that the terminal nucleotides of both strands are base paired, or generate staggered ends in which one of the two strands protrudes to give a short
30   (1-4 nt) single strand extension. In the case of restriction fragments with flush ends, adaptors are used with one flush end. In the case of restriction fragments with staggered ends, adaptors are used that have a single stranded extension complementary to the
35   single stranded extension on the restriction fragment.

Consequently, each type of restriction site end is
specifically recognized by a particular adaptor by
virtue of the complementarity of the matched ends. In
addition, the DNA sequence of the entire length of each

5   adaptor type differs from that of other adaptor types
(see Table I). Typically, the adaptors used are
comprised of synthetic single-stranded oligonucleotides
which are in part complementary to each other, and
which are usually approximately 10 to 30 nucleotides

10 long, preferably 12 to 22 nucleotides long, and which
form double stranded structures when mixed together in
solution. Using the enzyme T4 DNA ligase, the adaptors
are joined covalently and specifically to the
complementary ends of individual DNA molecules in the

15 mixture of restriction fragments generated from a
particular genomic DNA source. Using a large molar
excess of adaptors over restriction fragments ensures
that all restriction fragments will receive adaptors at
both ends. These adaptors are not usually

20 phosphorylated. These ligated adaptors then serve as
templates for the adaptor-directed PCR primers.
        In one embodiment of the invention, all
restriction fragments from the genome carry the same
adaptor at both ends, and a single PCR primer

25 corresponding to that adaptor sequence can be used to
amplify simultaneously from the fragments. The
simultaneous amplification of several different
restriction fragments is often referred to as multiplex
PCR amplification. Since in such a case all

30 restriction fragments are bordered at both ends by the
same adaptor, it is obvious that primer extension and
PCR amplification of a mixture of tagged restriction
fragments will amplify all restriction fragments in a
synchronous fashion. In another embodiment using two

35 or more different restriction enzymes to cleave the

DNA, two or more different adaptors are ligated to the
ends of the restriction fragments. In this case, two
different PCR primers, each matching the sequence of a
particular adaptor, can be used for exponential
5   amplification from a subset of the restriction
fragments. In one preferred embodiment using two or
more restriction enzymes, both adaptors are unmodified,
and the fragment mixture is enriched using a pre-
amplification step. In another preferred embodiment
10   using two or more restriction enzymes, the adaptor
corresponding to one of the restriction enzyme site
ends is covalently linked to a biotin molecule. Using
standard methods for isolating biotinylated molecules,
this design allows for the selection, from a complex
15   mixture of restriction fragments, of only those
bordered on one or both ends by a biotinylated adaptor.
Both of the two possible selection steps reduces the
complexity of the starting mixture of restriction
fragments and constitutes an enrichment step prior to
20   the PCR amplification, thereby reducing in certain
instances the background of fragments with same-site
ends. In yet another embodiment one of the
amplification primers may be radiolabeled for
identification of the products via autoradiogrpahy, or
25   may be modified with fluorescent tags for fluorescence
detection of products. Methods of labeling nucleic
acids and suitable labels are well known in the art
(see Sambrook *supra*). For example a radioisotope
suitable in the present invention is ^{33}Phosphate,
30   incorporated at the 5' end of one strand of the adaptor
by a phosphate group transfer from of [γ-^{33}P]ATP under
kinasing conditions.

Double stranded adaptors (with or without biotin
labels) are generated by annealing the two partially
35   complementary single stranded component

oligonucleotides of each pair (listed in Table I). For
example,the double stranded Taq I adaptor (Taq-Ad) is
produced by combining the single-stranded Taq.AdF and
Taq.AdR oligonucleotides under favorable annealing
5   conditions. To generate the biotinylated double
stranded Pst I adaptor (biotin-Pst-Ad), the single
stranded oligonucleotides biotin-Pst.AdF and Pst.AdR
similarly are combined. If restriction enzymes other
than those listed in Table I are used, then the
10   adaptors must be designed to carry the appropriate
protruding single-stranded-ends for a given restriction
1/2-site. In a preferred embodiment, each adaptor
contains a single base alteration within the
restriction half-site it carries, so that the
15   reconstructed site generated by each ligation event
cannot be re-digested. Therefore, the artisan will
appreciate that it is possible that restriction
digestion and ligation can be performed simultaneously
under the appropriate buffer and temperature
20   conditions.
DNA AMPLIFICATION:
        Although the basic protocols for the amplification
of nucleic acids are well known in this art,
significant modifications of those protocols were
25   necessary in order to achieve optimal amplification of
DNA fragments and detection of polymorphic products.
Several factors were found to significantly influence
these amplifications, including variation in the
thermocycling parameter of the PCR protocols, variation
30   in the labeling of the primers, and the length and
nucleotide composition of the primers.
Thermocycling variation in PCR:
        The efficiency of amplification by both 5'-
anchored simple and compound SSR primers was tested on
35   soybean, corn, and mammalian templates, using

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thermocycling profiles having either constant
temperature or touchdown annealing conditions. Both
the adaptors and the SSR-directed primers in these
amplifications were designed to have T_m's within a
5   relatively narrow range (38-45°C in 50 mM Na or K
salt), so that any primer pair chosen for an
amplification would have approximately the same optimal
annealing temperature.

Three different constant annealing temperatures,
10   52°C, 58°C and 60°C were tested using a standard 3-step
per cycle protocol. Although the results from each
test varied from the others (the higher the
temperature, the fewer the products generated), it is
clear that the efficiency of primer discrimination at
15   most target loci was found to be unacceptably
inefficient at any of these constant annealing
temperatures. Every "product" from these
amplifications was represented by a small family of
bands, and the products within each family differed by
20   multiples of two; the length of each dinucleotide
repeat. However, this "stuttering" effect and
potential nonspecific product formation was minimized
when a touchdown thermocycling protocol (Don, et al.,
Nuc Acids Res, 19, 4008 (1991)) was used. In touchdown
25   amplification, the annealing temperature begins
deliberately high, then is incrementally lowered in
successive cycles, down to a desired, "touchdown"
annealing temperature. Touchdown temperatures of 59°C,
58°C and 56°C were tested for some primer combinations.
30   For most SSR-directed primers, the 56°C final touchdown
conditions produced the greatest number of specific,
non-stuttering bands. For the purpose of the present
invention, therefore, it is most preferred if nucleic
acid amplification be conducted according to a
35   touchdown protocol where 56°C is the optimal final

annealing temperature. However, depending on the actual composition of the primers, a particular amplification may involve final annealing temperatures of 55°C-60°C.

5   Another variable in the thermocycling protocol that was explored is the method by which the amplification reactions are initiated. Typical PCR amplification protocols initiate with a cold start; all reagents necessary for DNA amplification are present in
10   the reaction mixture prior to the first denaturation step. In contrast, a hot start protocol calls for the exclusion of one key reaction component, typically either the primer, nucleotides or polymerase, from the mixture during reaction setup and the first
15   denaturation. This component then is added following denaturation, and primer extension can proceed.

        The cold start protocol allows for the possibility that primers will anneal under nonstringent conditions both to template sites that are not necessarily a
20   perfect match, and to multiple, staggered sites within a target locus. Often this method leads to a stuttering effect of the amplification products on the gel. In contrast a hot start protocol prevents spurious primer annealing to incorrect template sites
25   at ambient temperatures prior to the first denaturation, and generates products that resolve more sharply and discretely on the gel. When otherwise identical amplification reactions were performed using the cold start and hot start protocols, it was found
30   that for nearly every 5'-anchored simple SSR-directed primer, a cold start produced unacceptably indistinct products on the gel. Much sharper products were generated using hot start. In contrast, product resolution was found to be more consistent between cold
35   and hot start methods when using compound SSR primers.

In spite of the potential drawbacks in product
resolution, cold start amplification was easier to
perform, particularly when processing large numbers of
samples, and was routinely found to be sufficient to
5　generate amplification products that could be
distinguished as polymorphic between genomes.
Therefore, a slight gain in product resolution is
sacrificed in a cold-start protocol in exchange for
greater speed and ease of reaction setup. If
10　5'-anchored simple SSR primers are used, a hot-start is
preferred, but a cold-start is adequate and sufficient
for amplification reactions involving most compound
SSR-directed primers.

Choice of primer labeling:

15　　　The complementary strands of a duplex DNA molecule
usually resolve independently to slightly different
positions on a denaturing polyacrylamide gel. If both
strands of a DNA duplex are radiolabeled, then the
autoradiograph will show a separate band representing
20　each strand of each amplification product. Resolution
of only a single band for each amplification reaction
product on the denaturing polyacrylamide gel requires
that only one strand of each product be labeled. To
achieve this, only one of the two primers in any given
25　pair used for an amplification reaction should be
labeled. Either ^{32}P or ^{33}P can be used as radiolabels,
although ^{33}P images are generally sharper on an
autoradiograph. Alternatively, a variety of different
fluorophores can be incorporated into the primer; the
30　resulting products can then be detected using a
fluorescence detection system.

　　The effects of radiolabeling the SSR primer, in
comparison to the alternate labeling of the adaptor-
directed primer, were explored. In all protocol
35　variations, radiolabeling the adaptor-directed primer

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resulted in significant background. Generally, the
lanes on the gel contained a few discrete bands, but
the major result in every case was a smear of products
distributed along the entire length of the lane. In
5   contrast, when only the SSR-directed primer was labeled
in the amplification reactions, the products were much
more discrete on the autoradiograph and not associated
with any significant lane background. This difference
likely results from the high abundance of adaptor
10   target sites on a large proportion of the template
fragments (from which even linear amplifications with
labeled primers collectively will lead to significant
backgrounds). In contrast, the labelled SSR primers
have far fewer target sites in the template mixture,
15   and most become part of a productive, exponential
amplification. Hence 5'-labeling of only the SSR
primer is preferred, and would apply to labeling with
either radioactive or fluorescent tags.
Variations in SSR and adaptor primer design:
20   Variability in the SSR-to-adaptor amplification
reaction, and therefore in the products obtained,
results not only from the reaction and thermocycle
setup conditions described above but also from
subtleties in the design of the primers used in these
25   amplifications. Once a particular compound SSR has
been chosen as the target locus sequence for this
assay, then either partially or entirely different sets
of amplification products can still be controlled by
altering any one of the following primer design
30   criteria:

                    a)   the number and base composition of the
                          3'-extension nucleotide(s) on the
                          adaptor-directed primer;

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b) the relative lengths of the two
    constituent simple repeats that comprise
    the compound SSR primer;

c) the particular strand of the double-
5   stranded compound SSR locus chosen to
    correspond to the single-stranded primer
    (i.e., the directionality of the primer);

d) choice of restriction enzymes and SSR
    targets for a particular genome.

10   a)   Length of the adaptor-directed primer:

An adaptor-directed amplification primer which
corresponds to the sequence of one of the synthetic
adaptors ligated to the restricted ends of the genomic
DNA can carry a variable number of arbitrary sequence
15   nucleotides (zero to ten) at its 3'-end. These
variable 3'-nucleotides on the primer anneal
specifically to sequences that are directly adjacent to
the adaptor and restriction site and whose sequences
are not known, a priori, on any particular genomic
20   restriction fragment. The recognition of each such
primer to only a subset of all possible fragments in
the template mixture provides exquisite specificity in
the amplification reaction (Zabeau, EP 534,858). Such
primers, otherwise identical in sequence except for
25   differences in the few 3'-most nucleotide(s), can
amplify completely nonoverlapping sets of amplification
products and behave much like allele-specific
amplification primers (Newton et al., (1989) Nuc. Acids
Res 17: 2503; Kwok et al., (1990) Nuc. Acids Res. 18:
30   999; Wu et al., (1989) Proc. Natl. Acad. Sci. USA 86:
2757). The key difference, however, is that use of
these adaptor-directed primers requires no prior
sequence knowledge of the genomic locus to be
amplified, and each primer will selectively co-

recognize multiple target sites in a template DNA
mixture.

In general, the longer the variable 3'-extension,
the more selective or restrictive the primer. This
5   3'-extension contains arbitrary, nondegenerate or
partially degenerate bases, which restrict annealing of
the primer to only a subset of the total number of
potential target sites, thus leading to a reduction in
the real number of co-amplified products. The addition
10 of each nondegenerate nucleotide onto the 3'-extension
leads hypothetically to 4-fold greater template
discrimination. In addition, different single
nucleotides at the 3'-most base position(s) confer
unique template specificities to otherwise identical
15 primers. Thus, varying both the number and composition
of the 3'-selective nucleotides on the adaptor-directed
primer is sufficient to generate individual, either
partially or completely, nonoverlapping sets of
amplification products from the same template when
20 paired with a given SSR-directed primer. The choice of
which 3'-extension to use for a particular
amplification is largely a matter of chance, but still
will depend largely upon relative nucleotide
frequencies in a target genome and upon the abundance
25 in the genome of the specific SSR that serves as the
other priming site.

b) Relative lengths of the two constituent simple
repeats comprising the compound SSR primer:

Every simple and compound SSR locus in the genome
30 is a double stranded structure whose individual strands
carry different permutations of nucleotides. Unless
the core dinucleotides of a compound repeat are
palindromic (e.g., $(CA)_x(TG)_y$ or $(AG)_x(CT)_y$), a single-
stranded primer that may specifically anneal to one
35 strand at a particular SSR locus will not anneal to the

opposite strand. None of the in-phase compound SSRs is
palindromic and only 8 of the 90 possible dinucleotide
permutations represents such a palindrome. Therefore,
all in-phase and most out-of-phase SSR-directed primers
5   will primer-extend from each genomic target locus in a
polar, unidirectional manner, and any compound SSR
locus can be recognized and amplified from by any of
four primer classes. For example, the compound in-
phase SSR locus,

10   5'-CACACACACACACACACACACATATATATATATATATATATA-3' SEQ ID NO.:1

          3'-GTGTGTGTGTGTGTGTGTGTGTATATATATATATATATATAT-5', SEQ ID NO.:2

can be recognized by four different canonical primer
classes:

          5'-(AC)$_x$(AT)$_y$-3',
15   5'-(CA)$_x$(TA)$_y$-3',
          5'-(AT)$_x$(GT)$_y$-3',
          and 5'-(TA)$_x$(TG)$_y$-3', where the 5'-most
repeat in each serves primarily to anchor the primer to
the template, and the 3'-most repeat serves a primer-
20   extension function (see Figure 1c).

          Each of these four canonical primer classes can
include a wide range of individual primers, all
differing by the length of the two constituent repeats
within the primer. Changes in the lengths of these
25   constituent repeats have profound effects on primer
efficacy and the fidelity of reproducible
amplifications. In general, the longer the 5'-
anchoring repeat (i.e., the value of x, above) relative
to that of the 3'-priming repeat (the value of y), the
30   better the primer's specificity and priming efficiency
in the amplification. In addition, only a primer with
a short 3' repeat will allow amplification from
compound SSR loci containing very short downstream
repeats.

compound SSR loci containing very short downstream
repeats.

c) Polarity of the single stranded compound SSR-
    directed primer:

5   The choice of which strand of a double-stranded
compound SSR locus to use as a primer can be extremely
critical for determining the success of the SSR-to-
adaptor amplification reaction. It should be noted
that the only type of (AT)-containing primer that will

10   lead to efficiently generated amplification products
under standard conditions is one in which the $(AT)_n$
sequence is very short (1.5-3 repeat units) and is
situated as the 3'-primer extension end. An $(AT)_n$
repeat of any length at the 5' end is completely

15   inefficient as an anchor, and results in little or no
amplification from a complex genomic template mixture.

d) Restriction site frequencies, nucleotide bias, SSR
    frequencies, and primer design considerations:

        Ligation products carrying biotinylated adaptors

20   may be selected out of each digestion/ligation mixture
using a streptavidin or avidin coated support such as
paramagnetic beads, as provided by Dynal Inc.,(Lake
Success, NY). This selected DNA does not have to be
purified further from the beads for the subsequent

25   amplifications. In one embodiment where two
restriction enzymes are used and only the adaptor
corresponding to the restriction enzyme with the
hexanucleotide site is biotinylated, the selected DNA
is a mixture of fragments bordered only by one or the

30   other restriction site or flanked at each end by
different sites. For example, if Taq I and Pst I are
used, then only the Pst I adaptor is biotinylated.
Following biotin selection, the Taq I-Pst I and
Pst I-Pst I fragments are predicted to be present in

35   the enriched fragment mixture at an approximate ratio

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of 30:1, respectively. All Taq I-Taq I fragments are effectively discarded. Methods for such a calculation will be apparent to one skilled in the art, for example: if the frequency of each nucleotide is known

5 for the specific genome, then the symmetric and asymmetric restriction fragments will be present in the digestion mixture at predictable proportions. In general, a calculation can be made that derives from the following assumptions:

10   First, recognition sites for each restriction enzyme are present in the genome at differing absolute frequencies, which are a function of the number of nucleotides in the site and of the genome's nucleotide composition. Second, these absolute frequencies can be

15 converted to relative frequencies, p and q, since the sum of the relative frequencies (p+q) is always equal to 1. For example (considering equal nucleotide frequencies and random nucleotide distribution in a genome):

20 | site | absolute frequency | relative frequency |
|------|-------------------|-------------------|
| Taq I | $(0.25)^4 = 3.9 \times 10^{-3}$ | $p = 0.9412$ |
| Pst I | $(0.25)^6 = 2.44 \times 10^{-4}$ | $q = 0.0589$ |

Finally, the relative frequencies of restriction fragments bordered by these sites are simply the

25 products of the relative site frequencies for each fragment type. Therefore:

fragment	relative frequency
Taq I-Taq I	$p^2 = 0.8862$
Pst I-Pst I	$q^2 = 0.0035$
Taq I-Pst I and Pst I-Taq I	$2pq = 0.1109$

30   Therefore in this embodiment, utilizing restriction enzymes with 4- and 6-bp recognition sites and assuming no nucleotide bias, the biotin-selected DNA fragments represent only 11.1% of the genome.

35 Different restriction enzymes with different site

frequencies will lead to a greater or lesser proportion
of the genome represented in the mixture of selected
fragments. Using several restriction enzyme
combinations will ensure better coverage of the genome
5   than just a single enzyme combination.

The selected DNA fragments may be used as a pooled
template mixture for polymerase chain reaction
amplifications using one each of a primer corresponding
to one of the adaptors (the one that was not biotin-
10   selected) and a primer directed to a particular
5'-anchored simple or compound SSR sequence. One
skilled in the art will appreciate that a detectable
product will result from any single genomic template
fragment only when exponential amplification occurs
15   between the adaptor-directed primer and an oppositely
oriented SSR-directed primer (see Figure 1a). Multiple
amplification products are expected from each template
DNA mixture since the SSR and adaptor sequences are not
single-copy sites. The multiplex ratio (the number of
20   co-amplified products) of each amplification reaction
is affected by the absolute genomic copy number of a
specific SSR sequence, and can be adjusted
experimentally for a given SSR by altering either the
level of degeneracy of a simple SSR primer's 5'-anchor
25   or the number and quality of the nondegenerate
selective nucleotides at the 3' end of the adaptor-
directed primer. For example, assuming equal
frequencies for all four nucleotides in the genome, the
addition of each successive nondegenerate nucleotide
30   onto the 3'-end of the adaptor-directed primer leads to
a 4-fold reduction in the number of co-amplified
products from a given template mixture (see Figure 1b).
Therefore, an adaptor primer carrying zero selective
nucleotides at its 3' end (eg., Taq.AdF; see Table I)
35   will co-amplifiy 4 times as many templates as will a

primer with a single, nondegenerate 3'-nucleotide
(e.g., Taq.pr6, whose 3'-extension is -A). Similarly,
this primer will co-amplify 4 times as many template
fragments as a primer carrying 2 selective nucleotides,
5   and so on. In general, the degree of selectivity of
the adaptor-directed primer can be estimated using the
formula, $1/4^{2n}$, where n=the number of selective bases.
It should be cautioned that although convenient, this
simplified calculation does not take into account the
10   base composition of the genome, nor of the recognition
sites of the restriction endonucleases used to produce
the genomic fragments.

1. Although most DNA fragments in the bead-selected or
pre-amplified mixture should be bordered at one end by
15   the adaptor corresponding to the adaptor-directed
primer to be used in the PCR, only a subset of these
fragments are expected to carry an internal simple
sequence repeat region complementary to a particular
SSR primer. Thus, amplification products will be
20   generated and detected only from the subset of target
molecules that not only are flanked by the primer-
specific adaptor but also contain an internal repeat
sequence matching the SSR primer. It should be noted
that absolute frequencies for the different repeats can
25   vary widely within a species and are not accurately
known for most plant genomes. Preliminary studies
indicate that in soybean, $(AT)_n$ is at least twice as
abundant as $(CT)_n$, which in turn appears to be somewhat
more frequent than $(CA)_n$ (Morgante & Olivieri, *Plant J*
30   3. 175 (1992); Akkaya et al., (1992) *Genetics*,
132:1131). In general, it is estimated that one SSR
longer than 20bp exists in plant genomes once every
23-29kb, compared to a figure of 6kb in mammals (Wang
et al., *Theor. Applied Genetics*: 88, 1 (1994);
35   Morgante & Olivieri, *Plant J* (1993); Beckmann & Weber

Genomics 12.627 (1992)). Frequencies of compound SSR
sequences, however, have not been documented in the
literature.

A completely degenerate 5'-anchor on a simple SSR
primer should prime from every locus in the genome that
carries that particular SSR sequence. Any degree of
nondegeneracy introduced into the anchor will reduce
the potential number of genomic target sites, and
therefore the number of amplified fragments. In a
genome with no nucleotide bias, the complexity of the
co-amplified products is reduced by a factor of 4 for
every anchor position that is assigned a nondegenerate
nucleotide. Each self-anchoring compound SSR primer is
expected to anneal and prime from every matching
compound SSR locus in the biotin-selected fragment
mixture (providing each target SSR locus has a
sufficient length to allow complete hybridization by
the primer).

Detection of polymorphisms between phenotypically
related individuals:

Individual gel banding pattern differences of the
co-amplified fragments between different templates
(i.e., different genomes) indicate polymorphisms
between the source genomes. The amplification products
generated with any of the compound SSR-directed primers
are a mixture of polymorphic and nonpolymorphic
fragments. Compared to a conventional AFLP reaction
(EP 0534858), from which most of the polymorphisms
detected are dominant, Applicants' compound SSR-to-
adaptor multiplexed amplification method generates a
greater proportion of codominant polymorphisms.
Although many of the amplification products generated
by this scheme are nonpolymorphic between closely
related strains, the proportion of polymorphic products
increases between more distantly related lines. In

general, genomic polymorphisms can be detected using
the SSR-to-adaptor multiplexed amplification among
individuals from within a species as well as between
species; the greater the evolutionary distance between
5 the genomes being compared, the more polymorphisms
expected. Both dominant and codominant polymorphisms
can be detected. In either case, a polymorphism
revealed by this method may result from any one or a
combination of possible causes:

10   1)   One or both restriction sites bordering a
given genomic region are missing in one genome
(analogous to an RFLP, but here detected by a different
method). This may be visible either as a dominant or a
codominant difference between genomes;

15   2)   Insertion or deletion differences exist
between genomes, within the genomic fragment bordered
by common restriction sites (this should be visible as
a codominant polymorphism providing the amplification
distance is not too great for either allelic fragment);

20   3)   Length differences in the simple sequence
repeat between genomes can lead to codominant
polymorphic amplification products, generally differing
in length by multiples of the repeat unit;

          4)   Single base differences are present
25 between genomes in the region immediately adjacent to
the restriction site, such that the 3'-selective
portion of the adaptor-directed primer can discriminate
between dissimilar templates, in a manner analogous to
an allele-specific amplification.

30   Because of all these potential sources of
polymorphism, the information content on a per locus
basis for this type of multiplexed amplification assay
is very high. A simple estimate can be made for the
minimum number of nucleotide positions at a locus that
35 are informative (i e., at which a polymorphism may be

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detected). For templates digested with both a 4- and
6-bp cutter:

>   4 (within the 4 bp restriction site)
>   + 6 (within the 6 bp restriction site)

5   + 0 to 10 (sequence immediately adjacent to the
adaptor-specific restriction site)

>   + 0 to 30 (number of nucleotides within the SSR
assayable for length variability between genomes)

>   = 10 to 50 nucleotides per amplified locus may be

10   informative for producing a polymorphism between
individual genomes. The first three factors in this
sum result from single nucleotide variation (e.g.,
substitutions) between genomes, whereas the fourth
factor in the sum results from repeat length variation.

15   Although small insertions and deletions distributed in
the entire genome can contribution to the detection of
length variability in this as well as other genome
assays, the greatly increased probability for repeat
length variation at each SSR target locus results in an

20   "above background" level of length polymorphism
detectable in the products.

        In comparison, the information content for RFLP
is 8-12, for RAPDs is 16-18, and for a standard AFLP
assay is generally 10-15 nucleotides. Furthermore,

25   compared to conventional AFLP and RAPD technologies, a
larger proportion of the polymorphism assayable by this
SSR-to-adaptor amplification method is detectable as
codominant differences between genomes.

        SSR-based polymorphisms detected using this SSR-

30   to-adaptor, or SAMPL, amplification method can be
converted into more conventional and convenient single-
locus SSR markers. This conversion can be performed,
for example, if the multiplexed approach is used to
quickly screen through hundreds or thousands of

35   possible polymorphisms between genomes, and if it then

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is desirable to subsequently assay a chosen subset of
these polymorphisms either at a larger, more high
througput scale or in order to examine polymorphism at
these particular loci more quickly and nonisotopically.

5   This conversion process requires that the desired band
be excised from the SAMPL gel and then sequenced. From
the nucleotide sequence deduced for the unique sequence
flanking the SSR, a "locus-specific" primer can be
designed, which flanks and is oppositely oriented

10 towards the SSR. This unique primer can then be paired
with a general adaptor-directed primer and used to
amplify from the original fragment mixture. The
resulting adaptor-to-unique primer PCR product then can
be sequenced to discover the other unique flanking

15 sequence of the SSR, and the second locus-specific
primer can be designed. Finally, the two oppositely
oriented locus-specific flanking primers are used as a
pair to amplify the region spanning the desired SSR
locus in a target genome.

20   EXAMPLES

                     MATERIALS AND METHODS

        Restriction enzymes, ligases and polymerases used
in the following examples were obtained from BRL Life
Technologies (Gaithersburg, MD) or New England Biolabs

25 (Beverly, MA).

        The source of the soybean cultivars, Bonus and
soja PI 81762, was Theodore Hymowitz, University of
Illinois. All other soybean lines, including the
G. max cultivars wolverine, NOIR-1, N85-2176, Harrow,

30 CNS, Manchu, Mandarin, Mukden, Richland, Roanoke, Tokyo
and PI 54-60, and the G. soja accession PI 440-913,
were obtained from the USDA Soybean Germplasm
Collection, University of Illinois (Dept. Agronomy,
Turner Hall, Urbana, IL). The source of the Z. mays

35 inbred cultivars, B73 and Mo17, and the elite lines,

LH82, LH119 and LH204, was Holden's Foundation Seeds,
Williamsburg, IA.   The source of the *Z. mays* inbred
line, CM37, was Benjamin Burr, Brookhaven National
Laboratory, Upton, NY.   The AEC272 and ASKC28 *Z. mays*
5   lines were obtained from Dr. Denton Alexander,
University of Illinois.   Genomic DNA from five
different human sources, as well as from salmon and
mouse BABL/c, was purchased from commercial sources
(Sigma, St. Louis, MO or Clontech, Palo Alto, CA).
10   Reagents, buffers and protocols used for
restriction digests, ligations, 5'-end phosphorylation-
labeling of primers and PCR amplifications are given
below in Table III.

TABLE III

REACTION PROTOCOLS

TEMPLATE PREPARATION AND AMPLIFICATION REACTION SETUP

Restriction Digestion

Reagent	Stock	Final Conc	Amount Stock Used	Comments
Genomic DNA	High-quality	.0833 ug/ul	2.5 ug	should be of the highest quality
10X Buffer	10X 100 mM Tris-acetate	1X 10 mM	5 ul	compatible with most restriction endonucleases and with the subsequent ligation reaction
	100 mM Mg-acetate 500 mM K-acetate 50 mM DTT pH 7.5	10 mM 50 mM 5 mM		

Restriction enzymes

Taq I (4 bp recognition site)	10 units/ul	5 units/ug DNA	1.25 ul	neither should be methyl-sensitive
Pst I (6 bp recognition site)	10 units/ul	5 units/ug DNA	1.25 ul	4 bp recognition site
				6 bp recognition site
H2O			bring to 50 ul	

Adaptor ligation

Reagent	Stock	Final Conc	Amount Stock Used	Comments
Taq I-Ad	50 pmol/ul	.833 pmol/ul	1.0 ul	double strand adaptor for 4 bp enzyme
Biotin-Pst I-Ad	5 pmol/ul	.0833 pmol/ul	1.0 ul	5'-biotinylated adaptor for 6 bp enzyme
10X Buffer		1X	1.0 ul	same buffer as for restriction digests
ATP	10 mM	0.2 mM	1.2 ul	

| T4 DNA Ligase | 1unit/ul | 1 unit/reaction | 1.0 ul | |
| H2O | | | 4.8 ul | bring to 10 ul total |

Primer radiolabeling

Reagent	Stock	Final Conc	Amount Stock Used	Comments
SSR primer	50 ng/ul	5 ng/ul	3.0 ul	for 17-18mers, equal to ~25 pmoles
10X Kinase Buffer	10X 600mM Tris-Cl, 100mM MgCl2 150 mM DTT pH 7.8	1X 60mM, 10mM 15mM	3.0 ul	Contains no ATP. Verify buffer compatibility with the enzyme manufacturer's recommendations
[gamma-33P]ATP (3000 Ci/mmol)	10 uCi/ul	1.67 uCi/ul	5.0 ul	At 3000 Ci/mmol, equal to ~17 pmol. 32P also can be used, but with poorer gel resolution.
T4 polynucleotide kinase	10 units/ul	0.17 units/ul	0.5 ul	
H2O			bring to 30 ul	

PCR amplification

Reagent	Stock	Final Conc	Amount Stock Used	Comments
biotin-streptavidin selected template DNA			2 ul	2.5 ug input genomic DNA in a final 200 ul selected volume is sufficient for 100 PCR amplifications
Taq I adaptor directed-primer SSR-primer	50 ng/ul	1.5 ng/ul	0.6 ul (30 ng)	eg., Taq.pr1, Taq.pr2, Taq.pr3, etc.
33P-labeled SSR primer	5 ng/ul	0.25 ng/ul	1.0 ul (5 ng)	
unlabeled SSR primer	50 ng/ul	1.25 ng/ul	0.5 ul (25 ng)	30 ng total (labeled + unlabeled)

10X PEC Buffer	1X	2.0 ul	recommended by Perkin-Elmer Cetus for AmpliTaq polymerase (other buffer, containing ammonium sulfate, also are suitable)
100 mM Tris-Cl	10 mM		
15 mM MgCl2	1.5 mM		
500 mM KCl	50 mM		
pH 8.5			
dNTP mixture	0.2 mM each	0.8 ul	all four dNTPs in equimolar amounts
5mM each dNTP			
AmpliTaq polymerase	0.025 U/ul	0.1 ul	Stoffel fragment of Taq polymerase is not suitable, however, other high-temperature polymerases that carry both a $5' \to 3'$ polymerase and exonuclease activity are suitable
5 U/ul			
H2O		bring to 20 ul	

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Preparation of genomic template DNA mixtures:

     Genomic DNA was isolated from soybean (*Glycine max*) cultivars using a CTAB/chloroform extraction and CsCl/centrifugation method (Murry et al., *Nuc Acids*

5 *Res*, 8, 4321, 1980), and from corn (*Zea mays*) cultivars using a urea extraction miniprep method (Chen et al., in The Maize Handbook., M. Freeling and V. Walbot, eds., (1993) pp 526-527 New York).

     Purified genomic DNA was prepared for

10 amplification reactions in a manner similar to that described by Zabeau (EP 534,858), by complete restriction endonuclease digestion followed by or coupled with ligation of site-specific double-stranded adaptors. The restriction enzyme combinations used for

15 the following examples were either Taq I + Pst I or Taq I + Hind III (a combination of enzymes with tetra- and hexa-nucleotide recognition sites, respectively). Between 1 and 2.5 ug of high molecular weight genomic DNA was digested with 5 units/ug of Taq I in a 50 ul

20 volume at 65°C for approximately 3 hours in a buffer containing 10 mM Tris acetate, 10 mM magnesium acetate, 50 mM potassium acetate, 5 mM dithiothreitol, pH 7.5, then digested further in the same buffer with 5 units/ug of Pst I or Hind III at 37°C for 3 h

25 (Table III). The digestion products generated from each input genomic DNA were a mixture of symmetric fragments, bordered at both ends by either Taq I or Pst I (or Hind III) sites, and asymmetric fragments flanked by both a Taq I site and a hexanucleotide site.

30   Double stranded adaptors were generated by slowly annealing equimolar amounts of the two partially complementary single stranded component oligonucleotides of each pair (see Table III). The double stranded Taq I adaptor (Taq-Ad) at 50 pmole/uL

35 was produced by combining 5000 pmole each of Taq.AdF

and Taq.AdR single-stranded oligonucleotides with H_2O
to a final volume of 100 uL. For the 5 pmole/ul Pst I
and Hind III adaptors (biotin-Pst-Ad or biotin-Hind-
Ad), 500 pmole each of the corresponding single

5   stranded oligonucleotides for each were combined in a
final volume of 100 uL. To generate the double-
stranded molecules, all mixtures were incubated at
sequentially decreasing temperatures: 65°C for 15 min,
37°C for 15 min, room temperature for 15 min, then

10 finally at 4°C.

This section describes the method that utilizes
biotin-streptavidin selection for enriching the genomic
fragment mixture prior to the SSR-to-adaptor
amplification. To each completed double digestion was

15 added 10 uL of a mixture containing 1 unit T4 DNA
ligase, 0.2 mM ATP, and the two double-stranded
adaptors, Taq-Ad (50 pmole) and biotin-Pst-Ad or
biotin-Hind-Ad (5 pmole), each carrying a different
synthetic DNA sequence along its length and each

20 complementary at one end to the single-stranded
tetranucleotide or hexanucleotide overhangs on the
genomic fragments (Table III). These adaptor ligation
reactions were incubated at 37°C for 3 h. Each adaptor
contained a single base alteration within the half-

25 restriction site it carries, so that the reconstructed
site generated by each ligation cannot be re-digested.
Therefore, restriction enzyme digestions and ligations
can be performed simultaneously providing the
activities of all enzymes used share a common optimum

30 reaction temperature.

A subset of ligation products, all carrying a
biotinylated Pst I adaptor at least one end, was
selected out of each digestion/ligation mixture using
streptavidin coated paramagnetic beads (Dynal, Lake

35 Success, NY). For each selection, 10 uL beads, washed

once in 200 uL STEX (100 mM NaCl, 10 mM Tris-HCl, 1 mM
EDTA, 0.1% Triton X-100, pH 8.0) then resuspended in
150 uL STEX, was added to each ligation reaction, and
the mixtures incubated for one hour at room temperature
5   on a gently rocking platform. The DNA-adhered beads
then were selected out of each mixture using a magnetic
rack support; the supernatent was aspirated away, and
the beads were resuspended in 200 uL STEX. Four
additional cycles of bead selection, washing and
10 aspiration were performed. The final resuspension was
transferred to a fresh tube, and the DNA-adhered beads
selected in this final cycle were resuspended in 10 mM
Tris-HCl, 0.1 mM EDTA; pH 8.0 (100 uL for 1 ug input
DNA or 200 uL for 2-2.5 ug input DNA). This selected
15 DNA, which did not have to be purified further from the
beads, was a mixture of Taq I-Pst I (or Taq I-Hind III)
and Pst I-Pst I (or Hind III-HindIII) fragments,
present at an approximate ratio of 30:1, respectively.
The selected DNA fragments were used as pooled template
20 for polymerase chain reaction amplifications using one
each of a Taq I adaptor-directed primer and a simple
sequence repeat (SSR)-directed primer.

For the alternative method of enriching the
restriction fragment mixture prior to the final PCR
25 amplification, both restriction site specific adaptors
may be either biotin-modified or unmodified. Following
adaptor ligation to the restriction fragments, the
entire mixture is subjected to up to 16 individual
amplification reactions, each of which employs a pair
30 of individual adaptor-directed primers. One primer of
each pair corresponds to one of the adaptor sequences,
and the second primer to the second adaptor sequence.
In each case, the amplification primer carries a
single, randomly chosen nucleotide·at its 3'-most end.
35 Each primer pair will specifically amplify an

approximately 1/16th subset of the original genomic
fragment mixture. Any or all of the pre-amplified
product mixtures derived from a common restriction
fragment population then can serve as an enriched

5   template for the subsequent amplification between an
SSR-directed primer and primer representing either of
the two flanking adaptors. In this case, this adaptor
primer typcially carries 2, 3, or more arbitrary 3'-
nucleotides, with the nucleotide closest to the

10 restriction site perfectly matching the one nucleotide
used on the pre-amplification adaptor primer.

EXAMPLE 1

AMPLIFICATION USING 5'-ANCHORED SIMPLE SSR PRIMERS IN A
MULTIPLEXED SSR-TO-ADAPTOR AMPLIFICATION TO DETECT
15   POLYMPORPHISMS AMONG SOYBEAN CULTIVARS
Example 1 describes the use of primers
corresponding to simple SSR sequences flanked at the
5'-end by degenerate nucleotides, for the amplification
of adaptor tagged restriction fragments and the

20 subsequent detection of genetic polymorphisms among
soybean cultivars. Adaptor-directed primers used in
these amplifications are shown in Table I. The SSR
primers, herein termed 5'-anchored simple SSR primers,
are listed in Table IV.

25

TABLE IV

5'-ANCHORED SIMPLE SSR PRIMERS

Primer Name	Sequence(5'→3')/SEQ ID NO.	Length	Tm (50mM Salt)	%GC
HBH(AG)8.5	HBHAGAGAGAGAGAGAGGA/SEQ ID NO.:3	20	45.7°C	45.00
BHB(GA)8.5	BHBGAGAGAGAGAGAGAGAG/SEQ ID NO.:4	20	47.7°C	52.50
DVD(TC)8.5	DVDTCTCTCTCTCTCTCTCT/SEQ ID NO.:5	20	45.7°C	47.50
VDV(CT)8.5	VDVCTCTCTCTCTCTCTCTC/SEQ ID NO.:6	20	47.7°C	52.50
DBD(AC)7.5	DBDACACACACACACACA/SEQ ID NO.:7	18	41.8°C	47.20
BDB(CA)7.5	BDBCACACACACACACAC/SEQ ID NO.:8	18	44.1°C	52.80

HVH(TG)7.5	HVHTGTGTGTGTGTGTGT/SEQ ID NO.:9	18	41.8°C	47.20
VHV(GT)7.5	VHVGTGTGTGTGTGTGTG/SEQ ID NO.:10	18	44.1°C	52.80
CCGG(T)10	CCGGTTTTTTTTTT/SEQ ID NO.:11	14	23.4°C	28.60
GCGC(A)10	GCGCAAAAAAAAAA/SEQ ID NO.:12	14	23.4°C	28.60
BDBD(AC)6.5	BDBDACACACACACACA/SEQ ID NO.:13	17	39.5°C	47.00
BHBH(AG)6.5	BHBHAGAGAGAGAGAGA/SEQ ID NO.:14	17	39.5°C	47.00
VHVH(TG)6.5	VHVHTGTGTGTGTGTGT/SEQ ID NO.:15	17	39.5°C	47.00
CGG(CA)C6.5	CGGCACACACACACACA/SEQ ID NO.:16	17	44.3°C	58.80

Adaptor modified, biotin-selected genomic DNA from
the *Glycine max* strains, NOIR-1, N85-2176 and
wolverine, and the *Glycine soja* cultivar, PI 81762,

5   were prepared as described in the MATERIALS AND
METHODS. SSR primers were 5'-end labeled with ^{33}P by
combining fifty microcuries of [γ-^{33}P]ATP (New England
Nuclear) with 150 ng of primer and 5 units of T4
polynucleotide kinase in a 30 uL reaction (Table III).

10   After incubation at 37°C for 1 h, a 1 uL aliquot of
this labeled primer mixture (containing 5 ng primer)
was used directly in each amplification reaction.

All amplification reactions were performed
simultaneously by cold start initiation and were set up

15   together at room temperature, using a series of
reaction cocktails. The master mixture, containing the
four components common to all reactions, consisted of
(per reaction) 2.0 uL of 10X PEC buffer, 0.8 uL all
four dNTPs (5mM each), 13.0 uL H$_2$O, and 0.1 uL

20   (0.5 units) Amplitaq DNA polymerase (Perkin Elmer
Roche) (see Table III and MATERIALS AND METHODS). An
aliquot of this master mixture was then added to the
appropriate primers to make individual full-primer
cocktails; for each final amplification reaction,

25   15.9 uL of master mix was combined with 0.6 uL
unlabeled Taq I adaptor-directed primer (stock is
50 ng/uL), 0.5 uL unlabeled SSR primer, and 1.0 uL (at

5 ng/uL) ^{33}P-labeled SSR primer. The appropriate
biotin-streptavidin selected template DNA (2 uL each)
was distributed into 0.2 mL microamplification tubes
(Robbins Scientific, Mountain View, CA) and placed in
5   individual wells of a Perkin-Elmer 9600 multiwell
plate. Eighteen uL of the appropriate full-primer
cocktail then was added, to give all reactions a final
volume of 20 uL. This final combination of reaction
components was quickly completed and the amplification
10   reactions initiated on a Perkin Elmer 9600 thermocycler
with as little delay as possible using either a
constant 58°C annealing or a 58°C touchdown
thermocycling protocol:

Touchdown annealing
denature: 94°C, 3 min
1 cycle: 94°C, 30 sec
                       65°C, 30 sec
                       72°C, 1 min
11 cycles: 94°C, 30 sec
                       64.4°C, 30 sec for 1st cycle,
                       then decrease by 0.6°C per cycle
                       for the next 10 cycles to a final
                       58.4°C
                       72°C, 1 min
23 cycles: 94°C, 30 sec
                       58°C, 30 sec
                       72°C, 1 min

Constant temperature annealing
denature: 94°C, 3 min
35 cycles: 94°C, 30 sec
                       58°C, 30 sec
                       72°C, 1 min

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The completed amplification reactions were diluted
with an equal volume of formamide stop solution (98%
deionized formamide, 2 mM EDTA, 0.05% bromophenol blue,
0.05% xylene cyanol), heated to 94°C for 3 min, then
5   quickly chilled on ice.   2.5 uL of each was immediately
loaded onto a 4.5% or a 6% denaturing 30 x 40 x 0.4 cm
polyacrylamide gel (7M urea, 4.5% acrylamide:
N-,N-methylene bis-acrylamide [19:1], 100mM Tris-HCl,
80mM boric acid, 1 mM EDTA, pH 8.3) that was first pre-
10   run in the same tris-borate-EDTA buffer at 55W for 30
minutes. The loaded samples were electrophoresed at
55W (corresponds to ~1400-1500V, 35-40mA) for two
hours, then the gel was transferred to chromatography
paper, vacuum dried for 2 h at 80°C, and exposed to
15   Hyperfilm-MP (Amersham) or Biomax (Kodak) X-ray film
with an intensifying screen at -70°C for 2 to 7 days.

Figure 2 shows a comparison of the amplification
products using the 5'-anchored simple SSR-directed
primers, $DBD(AC)_{7.5}$, $HBH(AG)_{8.5}$, and $HVH(TG)_{7.5}$. In all
20   cases, the SSR primer was ^{33}P-labeled and used in
combination with each of two different Taq I adaptor-
directed primers, Taq.Ad.F and Taq.pr6, which carry
zero and one 3' selective nucleotide, respectively (see
Table I). Panel a shows the amplification products
25   generated using constant temperature thermocycling and
panel b shows the products from a touchdown protocol.
Several different 5'-anchored simple SSR-directed
primers have been tested, all of which are listed in
Table IV. In general, it was found that all such
30   simple SSR-to-adaptor amplifications required a 0-2
nucleotide extension on the 3' end of the adaptor-
directed primer to give a suitable number of co-
amplified products.

Regardless of the annealing temperature, the
35   constant temperature anealing protocol produced bands

80

on the gels that were extremely smeared and indistinct
for nearly every primer combination tested.   Raising
the annealing temperature from 56° to 58°-59°C resulted
in somewhat less smeariness (Figure 2a); the products
5   of individual loci generally are discernable. However,
the products are not discretely sized, and instead
showed a high degree of "stutter" on the gel. The
highest constant annealing temperature tested, 60°C,
produced relatively few bands for some primer
10   combinations, and no bands for others (data not shown).
These results indicate that the efficiency of primer
discrimination at most target loci is relatively
inefficient when the annealing temperature is held
constant throughout the thermocycling. Either the
15   primer does anneal, but at multiple positions within a
target locus (producing a stuttering effect), or it
does not anneal stably to generate a product. Although
an optimal constant annealing temperature for any
primer pair utlimately should be determined
20   empirically, it is likely that heterogeneously sized
amplification products still will result using this
thermocycling method.

In contrast, thermocycling using touchdown
conditions (Don., et al.,, *Nuc. Acid Res.*, 19, 4008,
25   (1991) are designed to lead to highly efficient target
locus discrimination and to minimize or eliminate
spurious priming by either of the primers in the
amplification. In touchdown amplification, the
annealing temperature begins deliberately high, then is
30   incrementally lowered in successive cycles, down to a
desired, "touchdown" annealing temperature. Touchdown
temperatures of both 59°C and 56°C were tested. For
most SSR-directed primers, this range of final
touchdown temperatures was optimal for producing a
35   large number of relatively discrete bands on the gels

(see Figure 2b for an example of the products resulting
from 58°C touchdown reactions; other data not shown),
and these bands were reproducible from experiment to
experiment.

5   Individual banding pattern differences in the co-
amplified fragments between different templates (i.e.,
different genomes) indicate polymorphisms between the
source genomes. The majority of the amplification
products generated by this scheme appear to be

10   nonpolymorphic between the two closely related *G. max*
strains, NOIR-1 and N85-2176; however, a greater number
of polymorphic products are seen between the more
distantly related *G. max*, wolverine and *G. soja*
PI 81762 cultivars. In addition, some polymorphisms

15   appear to be dominant (a band amplified from one
genome, but no apparent corresponding band from the
other), and a few are potentially codominant (bands of
similar but nonidentical size amplified from each
genome).

20   This analysis illustrates two important features
of the ability to fine tune and customize this assay.
First, the two different 5'-anchored simple SSR
directed primers, HBH(AG)$_{8.5}$ and DBD(AC)$_{7.5}$, when used
against a common set of genomic templates, produced

25   completely different amplification patterns. That
these patterns reflect distinctly different subsets of
amplification products is consistent with the idea (see
Figure 1) that different subsets of restriction
fragments from the genome are likely to carry different

30   SSR target sites. Each band of the gel is the result
of a productive amplification between a particular SSR
sequence oppositely oriented relative to a Taq I site.
Given the estimation for relatively large spacing
between SSR sequences of all types in the soybean

35   genome (Wang et al., *Theor. Applied Genetics*, 88, 1

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(1994); Morgante & Olivieri, *Plant J* 3, 175 (1993)), it
is unlikely that the SSR-to-Taq I site products derived
from the HBH(AG)$_{8.5}$ and DBD(AC)$_{7.5}$ primers in this
example cover any genomic loci in common.

5   A second feature illustrated by this example is
that for any chosen SSR primer, the fewer the number of
3'-selective nucleotides on the adaptor-directed
primer, the greater the number of co-amplified bands.
In general, it is expected that the mixture of products
10   generated using the n=0 version of the adaptor primer
is more complex than that for an n=1 primer, and this
product mixture is more complex than that for an n=2
primer, and so on. In this example, the Taq I adaptor-
directed primer, Taq.AdF carrying zero 3'-selective
15   nucleotides, consistently produced a greater number of
labeled reaction products when paired with a given SSR
primer, compared to primers carrying one selective
nucleotide (Taq.pr6 [n=1=A] or Taq.pr8 [n=1=c]). While
in theory, an increase in length of the 3'-extension
20   from n=0 to n=1 should decrease the number of amplified
fragments four-fold, it is difficult in practice to
quantitate the real difference, primarily because of
the great degree of general smeariness and poor
resolution of the products derived from 5'-anchored
25   simple SSR primers. Clearly, however, the greatest
number of bands, along with the highest levels of
background, is visible in the lanes representing
reactions amplified with Taq.AdF.

        Third, thermocycling conditions employing
30   touchdown conditions generally serve to reduce the
smearing within the lanes, and generally makes for
sharper product bands, in comparison to use of a
constant annealing temperature. However, these sharper
bands still are accompanied by a great degree of

stutter, which hinders precise comparison of
polymorphisms between lanes (genomes).

 In general, genomic polymorphisms can be detected
among individuals from within a species as well as

5 between species; the greater the evolutionary distance
between the genomes being compared, the more
polymorphisms are expected. Both dominant and
codominant polymorphisms are revealed. However, no
matter what the specific reaction thermocycling

10 condition, the use of 5'-anchored simple SSR primers in
an SSR-to-adaptor amplification is not ideal for
identifying new polymorphisms. Even when the annealing
conditions are carefully optimized, as in touchdown
thermocycling or in a hot start initiation (not shown),

15 the individual co-amplification products resolve on the
gels as rather smeary and indistinct. The high degree
of stutter apparent on the autoradiographic images
counteracts the clarity of the bands attainable using
conventional AFLP (Zabeau EP 534,858), and prevents

20 accurate identification of all but the most prominent
polymorphisms.

EXAMPLE 2

AMPLIFICATION USING PERFECT COMPOUND SSR PRIMERS
IN A MULTIPLEXED SSR-TO-ADAPTOR AMPLIFICATION TO

25   DETECT POLYMPORPHISMS AMONG SOYBEAN CULTIVARS

 Example 2 illustrates the use of perfect compound
SSR-directed primers in an SSR-to-adaptor amplification
method similar to that discussed in Example 1 as a
means to improve the resolution and increase the level

30 of polymorphism among the multiplexed amplification
products. All of the individual compound SSR primers
used for these amplifications are listed in Table V.

TABLE V

COMPOUND SSR PRIMERS

Primer Name	Sequence(5'→3')/SEQ ID No.	Length	Tm (50mM Salt)	%GC
Compound SSR-Directed Primers				
(AT) 3.5 (AG) 7.5	TATATATAGAGAGAGAGAGAGA /63	22	42.3°C	31.80
(TA) 7.5 (GA) 4.5	ATATATATATATATAGAGAGAGAG /64	24	40.3°C	20.83
(CT) 5 (AT) 7	CTCTCTCTCTATATATATATATAT /65	24	40.3°C	20.80
(CT) 7.5 (AT) 3.5	TCTCTCTCTCTCTCTATATATA /66	22	42.3°C	31.80
(CT) 7.5 (AT) 2	CTCTCTCTCTCTCTATA /67	19	41.6°C	42.00
(AT) 3.5 (GT) 6.5	TATATATGTGTGTGTGTGTG /68	20	40.5°C	35.00
(AT) 6.5 (GT) 4.5	TATATATATATATGTGTGTGTG /69	22	38.5°C	22.70
(AT) 8.5 (GT) 3.5	TATATATATATATATATGTGTGTG /70	24	38.6°C	16.70
(CA) 4.5 (TA) 7.5	ACACACACATATATATATATATAT /71	24	38.4°C	16.70
(CA) 6.5 (TA) 4.5	ACACACACACACACATATATATAT /72	22	40.4°C	27.27
(CA) 7.5 (TA) 2.5	ACACACACACACACACATATAT /73	20	40.5°C	35.00
(GT) 7.5 (AT) 2	TGTGTGTGTGTGTGTATAT /74	19	39.5°C	36.80
(GA) 7.5 (TA) 2	GAGAGAGAGAGAGAGAGATAT /75	19	41.6°C	42.10
(TC) 3.5 (AC) 5.5	CTCTCTCACACACACACA /76	18	42.9°C	50.00
(TC) 4.5 (AC) 4.5	CTCTCTCTCACACACACA /77	18	42.9°C	50.00
(TG) 4.5 (AG) 4.5	GTGTGTGTGAGAGAGAGA /78	18	42.9°C	50.00

(CA) 4.5 (GA) 4.5	ACACACACAGAGAGAGAG /79	18	42.9°C	50.00
(TC) 4.5 (TG) 4.5	CTCTCTCTCTGTGTGTGT /80	18	42.9°C	50.00
(GA) 3.5 (GT) 5.5	AGAGAGAGTGTGTGTGTG /81	18	42.9°C	50.00

Most of these compound SSR sequences are represented
either on sequenced soybean genomic clones that were
shown by hybridization to contain one of the
dinucleotide repeats that comprise the compound SSR (M.
5   Morgante and C. Andre, unpublished), or on cloned plant
and animal sequences that have been entered into public
DNA sequence databases (e.g., GenBank; see Table II).
      DNA templates were generated essentially as
described in Example 1, from the *G. max* cultivars
10   wolverine, NOIR-1, N85-2176, Harrow, CNS, Manchu,
Mandarin, Mukden, Richland, Roanoke, Tokyo, PI54-60,
and Bonus, as well as from *G. soja* accessions PI81762
and PI440.913. Following double digestion with either
Taq I + Pst I or Taq I + Hind III, ligation of double
15   stranded Taq I-Ad and biotin-Pst I-Ad (or biotin-Hnd
III-Ad) adaptors, and selection of fragments carrying
at least one biotinylated Pst I or Hind III end, the
amplification reactions were performed using a series
of reaction cocktails and a cold start setup, all as
20   described in Example 1. In all cases, only the
compound SSR primer was 5'-end labeled using
[γ-^{33}P]ATP. The amplifications were performed on a
Perkin Elmer 9600 thermocycler using a 56°C final
annealing temperature touchdown profile:
25

denature: 94°C, 3 min
1 cycle: 94°C, 30 sec
                 65°C, 30 sec
                 72°C, 1 min
11 cycles: 94°C, 30 sec
                 64.3°C , 30 sec for 1st cycle, then
                 decrease by 0.7°C per cycle for the
                 next 10 cycles to a final 56°C.
                 72°C, 1 min
23 cycles: 94°C, 30 sec

56°C, 30 sec

72°C, 1 min

Following electrophoresis on 6% denaturing
polyacrylamide gels in tris-borate-EDTA buffer
essentially as described in Example 1, the co-amplified
5 products were visualized by autoradiography after
intensifying screen enhanced exposure at -70°C to Kodak
Biomax X-ray film.

All of the compound SSR-directed primers listed in
Table V have been used in this protocol to detect
10 polymorphisms among different soybean cultivars. All
primers used represent perfect compound SSR sequences
in which one of the component nucleotides is "in-phase"
across the two adjacent dinucleotide repeats.
Surprisingly, not all of the primers listed in Table V
15 were equally effective at generating products, and not
all generated products with the same degree of
polymorphism, even when they were predicted to do so
based upon cloned sequence compilations (Table II).
The compound primer that produces the greatest number
20 of co-amplified fragments from the soybean genome is
$(CA)_x(TA)_y$ (where x and y are multiples of 0.5, but
each ≥ 1). Figure 3a shows the amplification products
from a $(CA)_{7.5}(TA)_{2.5}$ version of this primer, used in
·combination with Taq.AdF (containing zero 3'-selective
25 nucleotides) and Taq.pr8 (one 3'-nucleotide, -C). Also
shown in Figure 3a are the products from the compound
sequence primers $(TC)_{4.5}(TG)_{4.5}$ and $(CT)_{7.5}(AT)_{3.5}$, which
amplify only relatively few fragments even when the
Taq I primer is completely nonselective. This result
30 was surprising, since $(CT)_x(AT)_y$ sequences appear to be
the second-most abundant class of compound repeat on
isolated soybean clones (see Table II). As primers,
$(CA)_{7.5}(TA)_{2.5}$ and $(CT)_{7.5}(AT)_{3.5}$ differ primarily in

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the length of their $3'-(AT)_y$ repeat. The differing
efficiencies of these two primers in otherwise
identical amplification reactions may largely be a
function of the length of the "leading" $3'-(AT)_y$

5   sequence. In constrast, the extremely low number of
amplified products resulting from $(TC)_{4.5}(TG)_{4.5}$ is
probably the result of low copy number of this compound
repeat in the soybean genome (see Table II). Shown in
Figure 3b are the products generated using

10   $(TG)_{4.5}(AG)_{4.5}$, and $(TC)_{4.5}(AC)_{4.5}$, each in combination
with the same two Taq I adaptor-specific primers. An
intermediate number of products are amplified by each
of these two compound SSR sequences.

The amplification products generated with any of

15   the perfect compound SSR-directed primers are a mixture
of polymorphic and nonpolymorphic fragments. For
example, some of the products from the $(CA)_{7.5}(TA)_{2.5}$
primer are completely nonpolymorphic among all 15
different *G. max* and *G. soja* genotypes tested; however,

20   many of the products reflect either dominant or
codominant polymorphisms among these genomes. The
products amplified using $(CA)_{7.5}(TA)_{2.5}$ in combination
with either Taq.AdF(n=0) or Taq.pr8(n=1=c) from
Figure 3a were cataloged:

25

Band scored as:	AFLP[a]	SSR-to-adaptor amplification $(CA)_{7.5}(TA)_{2.5}$	
		+ TaqAdF[b]	+ TaqPr8
monomorphic	64	12	14
dominant[c]	34	44	40
codominant[d]	1	8	8
codominant + dominant[e]	3	8	16
TOTAL	102	72	78

% polymorphic products	37%	83%	82%
Expected heterozygosity[f]	0.32	0.44	0.43

[a]The Amplified Fragment Length Polymorphism assay on the same set of 15 soybean cultivars as used for the SSR-to-adaptor amplifications, using two paired adaptor-directed primers corresponding to the Taq I adaptor and Pst I adaptor, respectively (not shown)

[b]The TaqAdF lanes carry a high level of background, and only the most unambiguous polymorphisms were scored

[c]A band was scored as polymorphic in the indicated category if at least one of the 15 genotypes showed a difference from the others

[d]These scorings are very conservative, minimum estimates of the true incidence of codominant, allelic products; only the most obvious codominant relationships among the 15 template genotypes were scored. A more accurate measurement of the codominance frequency in these reactions requires analysis of the segregation of potentially allelic bands in a population derived from pairs of these genotypes

[e]A band could be scored as both codominant and dominant if it appears to be completely absent from at least one genotype and if it also was represented by at least two size variants in bands that were amplified in other genotypes

[f]Expected heterozygosity ($H=1-\sum p_i^2$,) for each band (locus) was calculated as the sum of the allele frequencies (p_i) for that locus; an average of H for every polymorphic locus could then be calculated

　　　　This set of amplification products does not represent the entirety of the $(CA)_{\geq 7.5}(TA)_{\geq 2.5}$ loci in the soybean genome. Within the biotin-selected subset

5　　of Taq I + Pst I double digested template fragments, amplification was limited to only those $(CA)_{\geq 7.5}(TA)_{\geq 2.5}$ genomic loci for which this SSR is within an amplifiable distance of and is oriented oppositely to a Taq I site, and for which no other Taq I site lies on

10　　the other side of the SSR, between the SSR and the "selectable" Pst I site (see Figure 1). The remaining $(CA)_{\geq 7.5}(TA)_{\geq 2.5}$ loci in the genome can be amplified from template DNAs constructed using different sets of restriction endonucleases. Figure 4 illustrates that

15　　when just one of the two restriction enzymes is changed

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(Pst I is replaced by Hind III as selectable enzyme
site), the pattern of amplified fragments is markedly
different from that produced with Pst I + Taq I.
Therefore, generating templates restricted with
5   differing combinations of restriction endonucleases, as
well as assaying each template preparation with a large
set of different compound SSR and adaptor primer
combinations, should allow the detection of a greater
proportion of the total number of polymorphic SSR loci
10   in any given genome.

In comparison to the low resolution of
amplification products generated by SSR-to-adaptor
amplification using 5'-anchored simple SSR primers (see
Example 1), the alternative use of compound SSR primers
15   allows for a much greater level of product resolution.
Each band/product is accompanied by far less stutter,
and the overall background in the lanes is noticeably
reduced, even when a cold start amplification is used
(compare Figure 2 to Figures 3a, 3b). This lower
20   amount of background permits good discrimination of
individual products, allowing the assignment of allelic
relationships among bands to be made with a greater
level of confidence. In some instances, a polymorphism
might arise from single nucleotide variation within the
25   region covered by the restriction site or the adaptor-
directed primer. Nevertheless, a number of the
polymorphisms (most of those categorized as codominant
or both codominant and dominant among the 15 cultivars
tested) appear to arise from variation in the length of
30   the compound SSR through which primer extension occurs;
these are recognizable as codominant polymorphisms
whose sizes differ by multiples of the unit length of
the repeat. The ability to include repeat length
variation as one type of polymorphism that is
35   identifiable by this assay allows for a higher level of

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polymorphism to be visualized from each amplification reaction. It is possible that this assay will allow for the calculation of genetic distances, both within and between species, with great efficiency. Evidence

5   for this comes from similarity estimates (not shown) made from the data shown in Figures 3a and 3b; distances calculated for pairings of *G. max-G. max* genotypes from this data are less than the calculated distances for *G. max-G. soja* pairings.

10   EXAMPLE 3

AMPLIFICATION USING PERFECT COMPOUND SSR PRIMERS IN A
MULTIPLEXED SSR-TO-ADAPTOR REACTION FOR GENETIC MAPPING

Example 3 illustrates the use of the SSR-to-adaptor amplification method to generate genetic

15   markers. To determine whether the polymorphic bands detected between *G. max*, Bonus and *G. soja*, PI 81762 are genetically heritable, and to determine whether these polymorphisms could have utility for genetic mapping, polymorphic products between these strains

20   were scored and mapped to the soybean genome.

Genomic template DNAs were made, as described in the MATERIALS AND METHODS, from 66 individuals at the F_2 generation from a cross involving the bonus and soja parents (from T. Hymowitz, U. of Illinois). For this

25   example, Taq I and Hind III restriction endonucleases were used for the template DNA double digestions. The digestions, adaptor ligations, and subsequent streptavidin-biotin selections were performed as described in MATERIALS AND METHODS.

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TABLE VI

F2 Individual Designation

Marker Name	Band Name	Parents PI81762	Bonus	1	2	4	6	7	8	9	12	13	14	15	16	17	18	19	20
301	94-23.p4	a	B	B	a	a	a	a	a	a	a	a	a	B	a	a	a	B	a
305	94-23.p1	a	B	a	a	a	a	B	a	a	B	a	a	a	a	a	a	a	B
307	94-23.p5	A	b	b	b	A	b	b	b	b	A	A	A	b	b	b	b	A	A
309	94-23.p9	A	b	b	b	b	b	b	A	b	b	A	b	A	A	b	b	A	A
320	94-23.p6	A	b	H	A	B	b	H	H	B	b	A	b	B	b	b	b	B	b
322	94-23.p3	A	B	b	a	B	B	A	B	b	H	H	A	b	A	H	A	A	H
323	94-23.p7	A	b	B	b	b	A	A	b	B	A	b	X	b	A	A	B	a	B
324	94-23.P8a	a	B	a	a	a	B	a	B	a	a	a	a	a	a	a	a	a	a
325	94-23.p8b	a	B	a	a	a	a	a	a	a	a	a	a	a	B	a	a	a	a
326	94-23.p10	A	B	a	b	b	B	B	B	a	a	a	a	b	B	a	a	a	a
329	94-23.p12	A	b	b	b	A	b	A	b	b	b	b	b	b	b	b	b	b	b
331	94-23.p13	A	b	b	b	b	A	A	b	b	A	b	A	b	b	A	b	b	A
333	94-23.p14	A	b	b	b	b	b	b	A	A	b	b	b	b	b	b	b	b	A

Marker Name	Band Name	PI81762	Bonus	21	22	25	27	28	29	30	31	33	34	36	37	38	39	40	41
301	94-23.p4	a	B	a	a	B	a	a	B	B	a	B	a	B	a	a	B	a	B
305	94-23.p1	a	B	a	a	a	B	B	a	a	a	a	a	a	a	B	B	B	a
307	94-23.p5	A	b	b	b	b	b	b	A	A	A	b	A	b	A	b	A	b	b
309	94-23.p9	A	b	b	A	b	A	b	b	b	A	A	b	A	b	b	A	A	b
320	94-23.p6	A	b	b	b	b	b	b	A	A	A	b	A	b	b	A	b	B	B
322	94-23.p3	A	B	B	H	H	b	B	B	H	b	H	b	B	B	A	b	b	B
323	94-23.p7	A	b	a	b	a	a	a	b	b	a	b	a	B	a	a	a	a	a
324	94-23.P8a	a	B	a	B	a	a	a	B	B	B	B	a	B	a	a	a	a	a
325	94-23.p8b	a	B	a	a	a	B	a	a	a	a	a	B	a	E	B	B	B	a
326	94-23.p10	A	B	a	A	b	b	b	a	a	a	a	b	a	a	a	a	a	a
329	94-23.p12	A	b	b	b	b	A	b	b	b	b	A	b	b	b	A	b	b	A
331	94-23.p13	A	b	b	A	b	A	A	b	b	A	b	A	b	b	b	A	A	A
333	94-23.p14	A	b	b	A	b	A	b	A	b	b	b	b	A	b	b	b	b	b

Marker Name	Band Name	PI81762	Bonus	42	43	44	45	46	47	48	49	50	51	52	53	54	56	57	58
301	94-23.p4	a	B	a	a	a	a	a	B	a	a	a	a	a	B	B	B	a	B
305	94-23.p1	a	B	a	B	a	a	B	a	a	a	B	B	B	a	a	a	a	a
307	94-23.p5	A	b	A	A	b	A	b	A	A	b	b	b	A	b	A	b	b	b
309	94-23.p9	A	b	A	b	b	b	A	b	b	b	b	b	b	H	b	b	A	b
320	94-23.p6	A	b	H	b	b	A	b	b	b	b	H	A	A	A	b	H	A	b
322	94-23.p3	A	B	H	H	A	B	H	A	A	H	b	A	b	b	E	a	H	A
323	94-23.p7	A	b	b	b	b	b	A	b	A	b	A	A	E	B	b	b	b	b
324	94-23.P8a	a	B	B	a	a	B	a	a	a	a	a	B	B	B	a	B	a	a
325	94-23.p8b	a	B	a	a	a	b	a	a	a	a	a	a	a	a	a	a	a	a
326	94-23.p10	A	b	a	a	B	A	a	B	a	a	a	A	b	A	b	B	b	a
329	94-23.p12	A	b	b	b	A	A	b	b	A	a	A	b	A	b	b	b	b	b
331	94-23.p13	A	b	b	b	b	A	b	A	b	b	b	b	A	A	b	b	A	b
333	94-23.p14	A	b	b	A	b	A	b	b	b	b	b	A	A	b	A	A	A	b

Marker Name	Band Name	PI81762	Bonus	59	62	64	68	74	76	77	78	80	82	83	84	85	86	91	96
301	94-23.p4	a	B	a	a	a	a	a	a	a	B	a	a	a	B	B	a	a	B
305	94-23.p1	a	B	a	B	a	a	B	a	a	B	a	a	a	a	a	a	a	a
307	94-23.p5	A	b	b	A	b	b	b	b	b	b	b	b	b	b	b	A	b	A
309	94-23.p9	A	b	b	b	b	b	b	b	A	b	b	b	b	b	b	b	b	b
320	94-23.p6	A	b	b	A	A	b	A	b	A	b	A	A	A	b	E	E	B	b
322	94-23.p3	A	B	H	b	A	H	H	H	A	B	A	A	A	E	b	A	B	H
323	94-23.p7	A	b	A	b	B	a	a	b	a	b	a	a	a	b	A	b	a	E
324	94-23.P8a	a	B	B	B	a	B	a	a	a	B	a	a	a	B	B	a	a	B
325	94-23.p8b	a	B	a	a	a	a	a	a	a	a	a	a	a	a	b	a	a	a
326	94-23.p10	A	b	b	b	A	b	b	A	b	B	b	A	A	A	b	b	b	A
329	94-23.p12	A	b	A	b	b	A	A	A	B	A	A	b	b	b	A	b	A	b
331	94-23.p13	A	b	b	b	b	b	A	A	b	b	A	A	b	A	A	b	b	A
333	94-23.p14	A	b	b	b	b	b	b	A	b	A	b	A	A	b	E	b	A	b

Marker Name	Band Name	PI81762	Bonus	Linkage Group	Log-Likelihood of Linkage
301	94-23.p4	a	B	LG19	16.9
305	94-23.p1	a	B	LG1	10.8
307	94-23.p5	A	b	LG11	11.4
309	94-23.p9	A	b	LG15	9.4
320	94-23.p6	A	b	LG6	10.4
322	94-23.p3	A	B	LG2	5.4
323	94-23.p7	A	b	LG10	5.8
324	94-23.P8a	a	B	LG14	12.0
325	94-23.p8b	a	B	LG13	11.3
326	94-23.p10	a	B	LG11	14.1
329	94-23.p12	A	b	LG9	3.6
331	94-23.p13	A	b	LG1	3.3
333	94-23.p14	A	b	LG5	11.7

The $(CA)_{7.5}(TA)_{2.5}$ compound SSR-directed primer
(Table V) was 5'-end labeled using $[\gamma\text{-}^{33}P]ATP$ and then
used in combination with Taq.pr6 adaptor-directed
primer (Table I) for multiplexed amplifications on the

5   parent and F_2 templates. These amplifications utilized
the cold start, 56°C touchdown thermocycling protocol
detailed in Example 2. Amplification products were
resolved on 6% denaturing polyacrylamide gels, then
dried and exposed to Kodak Biomax X-ray film. The

10 autoradiograph of these products is shown in Figure 5a.
At least 15 amplification products were decisively
polymorphic between the parental templates, and
demonstrated mendelian segregation among the F2
individuals. Most of these segregate as dominant

15 polymorphisms, and the probability that each segregated
in the F2 progeny at a 3:1 or a 1:2:1 mendelian ratio,
simply by chance alone, was consistently less than 5%.
The parental inheritence of each polymorphic product
was determined for each of the 66 F2 individuals, and

20 the scores for 13 of these polymorphisms are shown in
Table VI. With this specific primer combination, most
of the more unambiguous of the polymorphic bands appear
to segregate as dominant markers; only a few
polymorphisms appear to segregate codominantly. With

25 other specific primer combinations, however, the
incidence of codominant segregation is often higher.
Most codominant polymorphisms were more problematic to
score in that homozygotes sometimes could not be
distinguished from heterozygotes. Therefore, each of

30 the polymorphisms from this amplification were scored
with a default assumption of dominance, and instances
of true codominance were revealed following mapping.

In order to map these bands to the soybean genome,
these inheritence scores were correlated with those of

35 600 RFLP and single-locus SSR markers previously mapped

to the soybean genome (J. A. Rafalski and S. V. Tingey,
in: Genetic Maps: Locus Maps of Complex Genomes. 6th
Edition. Cold Spring Harbor Laboratory Press, Cold
Spring Harbor, NY, 1993). This standard genetic map

5   (data not shown) was constructed by standard RFLP
methodology from analysis of the segregation patterns
of many RFLP markers in the same F2 population as used
in this example (for standard RFLP mapping technology,
see T. Helentjaris, et al., 1986, *Theor. Appl. Genet.,*

10   72, 761 which reference is incorporated herein). The
basis for genetic mapping analysis is that markers
located near to each other in the genome are inherited
together in the F2 progeny, while markers located
farther apart are co-inherited less frequently.

15   Segregation analysis and marker map positions were then
calculated using a computer segregation analysis
program, MapMaker (E. S. Lander, et al., 1987 Genomics,
1, 174) which had been modified by Applicants for the
Macintosh. The results indicated that nearly every

20   polymorphic amplification product with an approximate
3:1 dominant segregation ratio, or a 1:2:1 codominant
segregation ratio, among the F2 progeny can be mapped
to the soybean genome. This is illustrated in
Figure 5b, where 6 polymorphisms from Table VI all map

25   to independent sites on various linkage groups. In
total, the 15 polymorphisms mapped from this single
primer combination are distributed among 10 different
linkage groups; in some instances, two or more
polymorphisms localize to linked sites on the same

30   linkage group. The probability that each of the
polymorphisms in Figure 5b localize to these positions
purely by chance from the observed data varied from 1
in $10^{3.29}$ to 1 in $10^{16.88}$, indicating the strength of
these map positions. This example demonstrates that

35   polymorphisms revealed by the present invention, SSR-

to-adaptor multiplexed amplification, have utility as
genetic markers.

DNA was isolated and templates were made from each
of 66 F2 individuals segregating from a cross between
5   PI81762 and Bonus soybean lines. The genotype of each
individual at each of the indicated marker loci was
determined as follows: A score of "A" or "B"
designates that the locus was inherited from the
PI81762 or Bonus parent, respectively. A score of H
10   designates that the locus was inherited from both
PI81762 and Bonus. A scope of "a" designates that the
locus was inherited only from PI81762 or that it was
inherited from both Bonus and PI81762. A score of "b"
designates that the locus was inherited only from Bonus
15   or that it was inherited from both Bonus and PI81762.
A score of "m" indicates missing data.

EXAMPLE 4
AMPLIFICATION USING PERFECT COMPOUND SSR PRIMERS IN A
MULTIPLEXED SSR-TO-ADAPTOR REACTION TO DETECT
20   ### POLYMORPHISMS IN OTHER PLANT AND ANIMAL GENOMES

Example 4 illustrates the use of perfect compound
SSR primers for the amplification of genomic DNA from
other non-soybean genomes including corn, salmon, human
and mouse. Genomic DNA was isolated from the *Z. mays*
25   inbred cultivars, B73, Mo17, ASKC28, LH82, LH119,
LH204, AEC272, and CM37, using a urea extraction
miniprep procedure (Chen et al., in: The Maize
Handbook., M. Freeling and V. Walbot, eds., (1993)
pp 526-527, New York). Genomic DNA from five different
30   human sources, as well as from salmon and mouse
(BALB/c) were purchased from commercial sources (Sigma,
St. Louis, MO or Clontech, Palo Alto CA). All these
DNAs were double digested either with Taq I + Pst I or
Taq I + Hind III, the restriction fragments ligated to
35   adaptors specific to these restriction sites, and

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biotin-streptavidin selections performed as described
in Examples 1 and 2.

Amplification reactions were performed as
described in Example 2, using several individual
5   perfect compound SSR-directed primers (^{33}P-labeled),
each primer in combination with individual Taq I
adaptor-directed primers carrying either zero or one
3'-selective nucleotide. The amplifications were
performed using cold start, 56°C final touchdown
10 thermocycling conditions, and the labeled products
resolved on 6% denaturing polyacrylamide gels.
Examples of the amplification products from these plant
and mammalian genomes are shown in Figure 6, panels a,
b and c.

15   In all genomes tested, every specific compound SSR
and Taq I adaptor primer combination produced a
distinct set of amplification products (a fingerprint).
In general, the degree to which any two fingerprints
are similar is a function of the evolutionary distance
20 between the individuals. These SSR-to-adaptor
amplification product fingerprints have two components
that can differ: the absolute number of fragments and
their collective pattern on the gel. First, a given
SSR-directed primer may generate a competely different
25 relative number of amplification products in one
species compared to another species, indicating that
the compound SSR locus amplified by that primer may be
present in entirely different copy numbers in one
phylogenetic group compared to another. For example,
30 the number of co-amplified fragments using the compound
repeat, $(CA)_{7.5}(TA)_{2.5}$ appears to be much greater (by at
least 2-5 fold) in the mammalian genomes than in
soybean or corn (see Figure 6) indicating that mammals
contain a greater number of $(CA)_{\geq 7.5}(TA)_{\geq 2.5}$ target
35 loci. This relationship is consistent with the greater

estimated fequency of $(CA)_n$ repeats in mammalian versus
soybean or corn genomes (Wang et al., *Theor. Applied
Genetics*: 88, 1 (1994); Morgante & Olivieri, *Plant J*
(1993); Beckmann & Weber *Genomics* 12.627 (1992)).

5   Second, within a narrow phylogenetic group (species or
genus), the pattern of amplification products between
individuals is generally similar, reflecting the
general conservation of restriction sites and SSR loci
in the different, yet closely related genomes.

10 Polymorphisms between individuals are detected whenever
a particular restriction site or SSR locus that
contributes to a given amplified fragment in one genome
carries a base substitution, insertion/deletion, or
repeat length difference compared to the other genomes

15 of the same species. Virtually no similarities are
obvious in the patterns of amplification products
between individuals whose evolutionary distance extends
beyond the same genus, consistent with the accepted
idea that the more diverged the two genomes being

20 compared, the more unlikely they will share common
loci. The sets of amplified fragments are entirely
different, for example, between individual genomes from
human compared to mouse or rat, and the amplification
patterns (and likely the set of amplified products)

25 appear to share no similiarities between soybean and
mammals, or even between soybean and corn.

EXAMPLE 5

EFFECTS OF VARYING THE PRIMER CONSTITUTION AND
THERMOCYCLING CONDITIONS TO GENERATE DIFFERENT
30   SETS OF AMPLIFICATION PRODUCTS

Viariability in the SSR-to-adaptor amplification
reaction, and therefore in the products obtained,
results not only from the reaction and thermocycle
setup conditions described above, but also from

35 subleties in the design of the primers and the

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thermocycling parameters used in these amplifications. Once a particular compound SSR has been chosen as the target locus sequence for this assay, then either partially or entirely different sets of amplification products still can be generated by altering any one of the following primer design criteria: 1) the number and composition of the 3'-extension nucleotide(s) on the adaptor-directed primer; 2) the relative lengths of the two constituent simple repeats that comprise the compound SSR primer; 3) the particular strand of the double-stranded compound SSR locus chosen to correspond to the single-stranded primer (i.e., the directionality of the SSR primer). In addition, the quality of the data generated by a particular amplification is affected by the mode by which thermocycling is initiated.

1. Design of the adaptor-directed primer:

This primer, which corresponds to the synthetic adaptor ligated to the restricted ends of the genomic DNA, can carry a variable number (zero to ten) and arbitrary sequence of nucleotides at its 3'-end. As described by Zabeau (EP 534,858), these variable 3'-nucleotides on the primer anneal specifically to "unknown" sequences that are directly adjacent to the adaptor and restriction site on a genomic DNA fragment, and the recognition by each of only a subset of all possible fragments in the template mixture provides exquisite specificity in the amplification reaction. Since such primers that are otherwise identical in sequence except for differences in the few 3'-most nucleotide(s) can amplify completely nonoverlapping sets of amplification products, they behave much like allele-specific amplification primers (Newton et al., (1989) *Nuc. Acids Res* 17: 2503; Kwok et al., (1990) *Nuc. Acids Res.* 18: 999; Wu et al., (1989) *Proc.*

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Natl. Acad. Sci. USA 86: 2757), except that their use
requires no prior sequence knowledge of the genomic
locus to be amplified, and each primer will selectively
co-recognize multiple target sites in a template DNA
5   mixture.

In general, the longer the 3'-extension the more
selective the primer; as the variable 3'-extension is
made longer, the adaptor primer becomes more restricted
to recognize a smaller number of potential genomic
10   target sites, leading to a smaller real number of co-
amplified products. The addition of each nondegenerate
nucleotide onto the 3'-extension leads to approximately
4-fold greater template discrimination. In addition,
different single nucleotides at the 3'-most base
15   position(s) give unique template specificities to
otherwise identical primers. These principles are
illustrated by the examples shown in Figure 7. The
^{33}P-labeled perfect compound SSR primer, (CA)$_{7.5}$(TA)$_{2.5}$,
was used for SSR-to-adaptor amplification to generate
20   specific products from the genomes of several soybean
cultivars, using the experimental conditions described
in Example 2. This SSR primer was paired with each of
several different Taq I adaptor-directed primers, which
all differ only at their 3'-most nucleotide positions
25   (see Table I):

.AdF	.pr5	.pr6	.pr7	.pr8	.pr9
0	1 (-G)	1 (-A)	1 (-T)	1 (-C)	2 (-AC)

The Taq I adaptor-directed primer with the shortest 3'-
extension (zero nucleotides), Taq.AdF, is completely
30   nonselective and generated the largest number of
products; the primer with the longest extension
(Taq.pr9) is the most selective and resulted in the
fewest amplification products. All four of the primers

carrying one nucleotide as a 3'-extension amplified
approximately the same number of products; however, the
set of products from each of these four primers is
unique compared to the sets from the other three. The
5   complex pattern of bands amplified using Taq.AdF is
actually a composite of all four one-base extension
primers. That is, the pattern generated by each of
these four primers is approximately a 1/4 subset of the
pattern amplified by the zero-base extended primer.
10   Thus, varying both the number and composition of the
3'-selective nucleotides on the adaptor-directed primer
is sufficient to generate individual, either partially
or completely, nonoverlapping sets of amplification
products from the same template when paired with a
15   given SSR-directed primer. The choice of which 3'-
extensions to use will depend largely upon relative
nucleotide frequencies in a target genome and upon the
abundance in the genome of the specific SSR that serves
as the other priming site.

20   2. Relative lengths of the two constituent simple
         repeats comprising the compound SSR primer:
         Every simple and nearly every compound SSR locus
in the genome is a double stranded structure whose
individual strands carry different permutations of
25   nucleotides. A single-stranded primer that may
specifically anneal to one strand at a SSR locus will
not anneal to the opposite strand (with the exception
of the 9 specific palindromic compound SSR sequences
combinations; see Table II). Therefore, a given SSR-
30   directed primer will primer-extend from each genomic
target locus in a polar, unidirectional manner, and any
compound SSR locus can be recognized and primed from by
any of four different primer classes. In addition,
each of these four canonical primer classes can include
35   a wide range of individual primers, all differing by

the length of the two constituent repeats within the
primer. Changes in the lengths of these constituent
repeats have profound effects on primer efficacy and
the fidelity of reproducible amplifications; in
5   general, the longer the 5'-anchoring repeat relative to
the 3'-priming repeat, the better the primer's
specificity and priming efficiency in the
amplification.

Multiple, individual primers, each differing from
10   the others by the length of its two constituent
repeats, have been tested for four different compound
SSR sequences: $(CT)_x(AT)_y$, $(AT)_x(AG)_y$, $(CA)_x(TA)_y$,
$(AT)_x(GT)_y$ (i.e., the values of x and y are varied for
a particular SSR type). Figure 8 shows the results of
15   a test using three different $(CA)_x(TA)_y$ primers,
$(CA)_{4.5}(TA)_{7.5}$, $(CA)_{6.5}(TA)_{4.5}$, and $(CA)_{7.5}(TA)_{2.5}$, and
three different $(AT)_x(GT)_y$ primers, $(AT)_{3.5}(GT)_{6.5}$,
$(AT)_{8.5}(GT)_{2.5}$, and $(AT)_{6.5}(GT)_{4.5}$. All five of these
primers were calculated to have a Tm in the range of
20   38-42°C. Each was 5'-labeled with ^{33}P and paired
individually with three different Taq I-directed
primers (Taq.AdF. Taq.pr6 and Taq.pr8) in amplification
reactions using biotin-selected soybean template DNAs,
PI81762 and wolverine, as described in Example 2.
25   Neither of the $(AT)_x(GT)_y$ primers performed very
efficiently, although $(AT)_{3.5}(GT)_{6.5}$ generated at least
some products, whereas the other two $(AT)_x(GT)_y$ primer
versions failed completely to generate any
amplification products. In contrast, all three
30   $(CA)_x(TA)_y$ primers were able to generate products,
although the number of amplified fragments varied among
the three primers. These results demonstrate that the
longer the 5'-anchoring repeat and the shorter the 3'-
primer extension repeat, the more amplification
35   products are produced. This same conclusion was drawn

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from similar experiments performed with $(CT)_x(AT)_y$ and $(AT)_x(AG)_y$ primers carrying variable constituent repeat lengths (not shown).

3.   Polarity of the single stranded compound SSR-
5   directed primer:

The choice of which strand of a double-stranded compound SSR locus to use as a primer can be extremely critical for determining the success of the SSR-to-adaptor amplification reaction. For some compound
10 SSRs, one strand of the double-stranded SSR was found to serve as an efficient primer whereas the opposite strand failed completely, regardless of the relative lengths of the constituent repeats on the primer. This difference was most extreme for compound SSRs
15 containing a $(AT)_n$ repeat; the only type of $(AT)_n$ containing primer that will lead to efficiently generated amplification products under standard conditions (described in Example 2) is one in which the $(AT)_n$ sequence is very short (1.5-3 repeat units) and
20 is situated as the 3'-primer extension end. Figure 8, for example, illustrates the superior efficiency of $(CA)_x(TA)_y$ primers in contrast to $(AT)_x(GT)_y$ primers, which represent the complementary strands of the same compound SSR. All three of the $(AT)_x(GT)_y$ primers
25 tested were extremely inefficient at generating amplification products (two were failures), even though the calculated T_m values of all the primers were approximately the same. It is likely that this difference in primer efficiency results from the
30 difference in placement of the $(AT)_n$ stretch within the primer. A and T nucleotides display weak hydrogen bonding during base pairing, and oligonucleotides containing $(AT)_n$ stretches often have self-complementarity artifacts in competition with weak
35 annealing to the template. Therefore, it is likely

than an $(AT)_n$ stretch at the 5' end of a primer will
serve poorly to anchor the primer to the template,
whereas a short $(AT)_n$ at the 3' end will have been
well-anchored by the upstream non-$(AT)_n$ portion of the
5   primer.

Primers corresponding to compound SSRs that are
devoid of $(AT)_n$ repeats are affected much less by the
relative order of the two constituent repeats. For
example, the individual primers in the two
10 complementary primer sets, $(TC)_{4.5}(AC)_{4.5}$ versus
$(TG)_{4.5}(AG)_{4.5}$ (see Figure 3) and $(CA)_{4.5}(GA)_{4.5}$ vs.
$(TC)_{4.5}(AC)_{4.5}$, (not shown) appear to generate
amplification products with approximately equivalent
efficiencies.

15 <u>4. Mode of thermocycling initiation</u>

The reaction setup protcol described in the
previous examples is essentially a cold start, and
allows the possibility for primers to anneal under
nonstringent conditions both to template sites that are
20 not necessarily a perfect match, and to multiple,
staggered sites within a target locus (the latter leads
to a stuttering effect of the amplification products on
the gel). This simple reaction setup protocol,
nevertheless, routinely was sufficient to generate
25 amplification products that could be distinguished as
polymorphic between genomes. In fact, the cold start
reaction products from coumpound SSR directed primers
were consistently quite sharp and distinct; however,
comparable products derived from 5'-anchored simple SSR
30 primers generally lacked the same clarity and sharpness
(compare Figures 2 and 3). Much of this indistinctness
and individual product heterogeneity, for both types of
SSR-directed primer, could be obviated by the use of a
hot start initiation for the thermocycling. A hot
35 start protocol (Chou et al., *Nuc. Acids Res.* 20, 1717,

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1992) prevents spurious primer annealing to incorrect
template sites prior to the first denaturation, and
generates products that resolve more sharply and
discretely on the gel.

5   Otherwise identical amplification reactions were
performed using the cold start procedure described in
Examples 1 and 2, and also using two different hot
start initiation procedures. For one type of hot
start, all the components of each amplification

10   reaction were combined as described in MATERIALS AND
METHODS, except that the SSR-directed primer (both 5'-
end labeled and unlabeled versions) was excluded from
the full primer cocktail. The reaction tubes were
capped (18.5 uL reaction volume), and the first

15   denaturation step of the thermocycling was performed
(94°C, 3 min). The reactions were then held at 80°C
while 1.5 uL of the appropriate SSR primer (1.0 uL of
5 ng/uL ^{33}P-labelled plus 0.5 uL of 50 ng/uL
unlabelled) was added to each reaction. Exponential

20   amplification was then initiated, using either a
constant 58°C annealing temperature or a touchdown (56°
or 58°C final) thermocycling protocol. For the second
hot start method, Ampliwax PCR-50 gems were used
essentially as described by the manufacturer

25   (Stratagene, La Jolla, CA), except that a mixture of
10X buffer, $MgCl_2$, dNTPs, H_2O, and primers (5.4 uL
total) was first heated with the Ampliwax and allowed
to cool, then a second mixture (14.6 uL) consisting of
template DNA, AmpliTaq DNA polymerase, PCR buffer and

30   H_2O was added over the top of the re-solidified wax
layer (final concentrations of each match those in the
standard cold start amplifications). Thermocycling was
then initiated, using the touchdown annealing protocol
detailed in Example 2.

The amplifications illustrated in Figures 2-8 all
employed a cold start protocol. A direct comparison of
the two initiation procedures, however, is shown in
Figure 9. Two sets of amplifications using the perfect
5 compound SSR primer, $(CA)_{7.5}(TA)_{2.5}$, paired with three
different Taq I adaptor primers (Taq.AdF, Taq.pr6,
Taq.pr8) were performed using the conditions described
in Example 2 (56°C final touchdown temperature); one
set was initiated with a standard cold start, the other
10 with the first hot start protocol described above.
These results demonstrate that a hot start is superior
for generating the most discrete bands with the least
amount of stutter, although the cold start is adequate
for producing products that nonetheless are discernable
15 as polymorphic between genotypes.

In general, a hot start produced the sharpest
product bands on the gel for nearly every SSR-directed
primer tested. The most extreme difference between
these protocols were observed when 5'-anchored simple
20 SSR primers were used. In fact, a cold start using 5'-
anchored simple SSR primers often led to unacceptably
smeared and fuzzy product bands. The differences were
generally more subtle for compound SSR primers,
although some SSR primers (those with long stretches of
25 $(AT)_n$ as the 5'-anchor) failed to produce any product
under hot start conditions.

<u>EXAMPLE 6</u>
<u>AMPLIFICATION USING PERFECT COMPOUND SSR-DIRECTED</u>
<u>PRIMERS FOR INTER-SSR AMPLIFICATIONS</u>
30   <u>TO DETECT POLYMORPHISMS</u>
Example 6 demonstrates the use of perfect, in-phase
compound SSR primers in a single-primer amplification
for the production and detection of genetic
polymorphisms. Simple SSR sequences containing 3
35 degenerate bases at the extreme 5'-end have been shown

previously to serve as efficient primers for
amplification between neighboring SSR sequences in the
genome (Zietkiewicz, et al., *Genomics*, 20, 176,
(1994)). Similarly, primers corresponding to compound
5   SSR sequences also are useful and extremely efficient
for generating inter-SSR amplification products in a
single-primer PCR reaction. Generally, the same
compound SSR primers that are most efficient in SSR-to-
adaptor amplifications, those with in-phase sequences
10 and corresponding to the most abundant of the compound
SSRs in the genome (see Table II) also are the most
efficient for generating inter-SSR amplification
products.

   Figure 10 illustrates the single-primer
15 amplification products obtained using both 5'-anchored
simple SSR primers and compound SSR primers, from three
different cold start thermocycling protocols: 58°C
constant, 58°C touchdown, and 56°C touchdown (described
in Examples 1 and 2). SSR primers were 5'-end labeled
20 with [γ-^{33}P]ATP as described in MATERIALS AND METHODS.
Either 20 ng undigested genomic DNA or the standard
amount of digested, biotin-selected adaptor modified
template DNA were combined with the compound SSR primer
(5 ng labeled primer combined with 25 ng unlabeled
25 primer) and the other non-primer components of the
amplification reactions described in the previous
examples. No adaptor-directed primers were added.
These reactions were performed using both hot start
(not shown) and cold start conditions although the
30 products resulting from hot start were more discrete
and better resolved on the gels.

   Figure 10, panel *a* shows a comparison of the
products from undigested genomic DNA for soybean (Bonus
and PI 81762) and corn (B73 and CM37) cultivars using
35 the 5'-anchored simple SSR primer, DBD(AC)$_{7.5}$,

generated with the three thermocycling profiles
indicated.   Panel b illustrates a comparison of the
amplification products obtained using the 5'-anchored
simple SSR primers, DBD(AC)$_{7.5}$ and HBH(AG)$_{8.5}$, and

5   perfect compound SSR primers, (AT)$_{3.5}$(AG)$_{7.5}$ and
(AT)$_{3.5}$(GT)$_{6.5}$, from both undigested and Taq I + Pst I
digested, biotin-selected template DNAs from soybean
wolverine and PI 81762. Two different thermocycling
methods were used, as indicated.

10   The undigested DNA templates produced a greater
number of amplification products than did the digested
templates. The reactions using digested, adaptor-
ligated templates served as single-primer controls for
the SSR-to-adaptor amplifications described in

15   Examples 1 and 2; relatively few fragments were
amplified by the single SSR primer from these cut DNA
templates, indicating that most of the amplification
products observed in the SSR-to-adaptor reactions are
dependent upon the presence of both the SSR sequence

20   and a neighboring adaptor sequence on each digested DNA
fragment. The few single-primer products that are
visible may result either from bona fide inter-SSR
amplification within single DNA fragments, or perhaps
from some sort of inter-fragment pairing.

25   These results demonstrate that compound, as well
as simple, SSR primers can generate inter-SSR
amplification products from the corn and soybean
genomes. The multiple products generated from an
individual SSR primer are a mixture of nonpolymorphic

30   and polymorphic fragments. The polymorphic bands
between genotypes indicate length differences between
or within neighboring SSR sequences in the genome;
these fragments are potential markers for genome
identification, fingerprint analysis, or marker

35   assisted selection.

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EXAMPLE 7

CONVERSION OF SSR-TO-ADAPTOR BAND POLYMORPHISMS TO SINGLE-LOCUS SSR MARKERS

Once a good SSR is found by using the SSR-to-
5   adaptor amplification (or SAMPL) method, it often may
be desirable to focus only on this single SSR, or just
a few SSRs, for subsequent analysis of a species, and
to examine variation at these few SSRs using a more a
straightforward, nonradioactive, single-locus method.

10   To accomplish conversion of a band from a SAMPL
gel to a single locus marker, this band is first
excised from the dried gel (see Figure 11). The DNA
from the band is eluted from the gel by heating in
100uL H_2O at 95°C, 15 min. The debris is pelleted by

15   centrifugation for 2 min at 12000 rpm, and the DNA in
the supernate is precipitated by adding 0.1 volume 3M
sodium acetate, pH 5.3, 0.025 volumes 20mg/mL glycogen
and 2.5 volumes ethanol, incubating at -70°C 30 min,
then centrifuging at 12000rpm, 10 min. The pelleted

20   DNA is washed once in 70% ethanol, air-dried, then
resuspended in 10uL H_2O. One uL of this DNA then is
used as template for PCR amplification using conditions
as described in Example 2, except that the SSR-directed
primer and the adaptor-directed primer (each at

25   1.5ng/uL final concentration; corresponding exactly to
the primer pair used for the original amplification)
each are unlabeled (see Figure 11). These
re-amplification products are purified using a Qiagen
(Chatsworth, CA) PCR fragment cleanup kit, and the

30   purified DNA fragments either are subcloned into a
suitable T-vector (for example, pGEM-T, Promega,
Madison, WI) and the insert sequenced using vector-
directed primers, or are sequenced directly without
subcloning, using the adaptor-directed primer as the

35   sequencing primer. In either case, the DNA sequence for

the amplified fragment is obtained, allowing design of
the first locus-specific flanking primer (lsfp-1).
This primer corresponds to the unique sequence flanking
the SSR on the amplified fragment, and is oriented with
5   its 3'-end toward the SSR (Figure 11).

The lsfp-1 primer then is paired with a primer
corresponding to the second adaptor used for the
initial preparation of the restriction fragment
templates, for amplification across the targeted SSR.
10   This amplification can be performed with or without
radiolabeling the lsfp-1 primer, although the
nonspecific background is reduced with a radiolabled
lsfp-1 primer. The specifically amplified adaptor-to-
lsfp-1 band is excised from the gel and sequenced as
15   described above. From the resulting DNA sequence of the
other flanking region of the SSR, a second locus-
specific flanking primer, lsfp-2, then is designed.
The 3'-end of this primer is oriented toward the SSR,
oppositely to lsfp-1.
20   Finally, the lsfp-1 and lsfp-2 unique primers are
paired and used for PCR amplification either using
restriction fragmented DNA template mixtures or using
unrestricted genomic DNA templates. Use of this primer
pair generates a locus-specific marker that spans the
25   targeted SSR. However, repeat length variation at this
SSR now can be detected quickly and nonradioactively
from any undigested genome using these specific
flanking region primers.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i)   APPLICANT: MORGANTE, MICHELE
                        VOGEL, JULIE M.

    (ii)   TITLE OF INVENTION: COMPOUND MICROSATELLITE
                                    PRIMERS FOR THE
                                    DETECTION OF GENETIC
                                    POLYMORPHISMS

    (iii) NUMBER OF SEQUENCES: 89

    (iv)   CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: E. I. DU PONT DE NEMOURS AND
                            COMPANY
        (B) STREET: 1007 MARKET STREET
        (C) CITY: WILMINGTON
        (D) STATE: DELAWARE
        (E) COUNTRY: U.S.A.
        (F) ZIP: 19898

    (v)   COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: FLOPPY DISK
        (B) COMPUTER: IBM PC COMPATIBLE
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PATENT IN RELEASE #1.0,
              VERSION 1.25

    (vi)   CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
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        (A) APPLICATION NUMBER: 08/346,456
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    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: FLOYD, LINDA AXAMETHY
        (B) REGISTRATION NUMBER: 33,692
        (C) REFERENCE/DOCKET NUMBER: BB-1064-A

    (ix)   TELECOMMUNICATION INFORMATION:
        (A) TELEPHONE: 302-892-8112
        (B) TELEFAX: 302-992-7949

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(2) INFORMATION FOR SEQ ID NO:1:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 40 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CACACACACA CACACACACA CATATATATA TATATATATA   40

(2) INFORMATION FOR SEQ ID NO:2:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 40 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TATATATATA TATATATATG TGTGTGTGTG TGTGTGTGTG   40

(2) INFORMATION FOR SEQ ID NO:3:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 17 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGAGAGAGAG AGAGAGA   17

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(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 17 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GAGAGAGAGA GAGAGAG   17

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 17 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCTCTCTCTC TCTCTCT   17

(2) INFORMATION FOR SEQ ID NO:6:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 17 base pairs
       (B) TYPE: nucleic acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCTCTCTCT CTCTCTC   17

(2)   INFORMATION FOR SEQ ID NO:7:

      (i)   SEQUENCE CHARACTERISTICS:
         (A)   LENGTH: 15 base pairs
         (B)   TYPE: nucleic acid
         (C)   STRANDEDNESS: single
         (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACACACACAC ACACA   15

(2)   INFORMATION FOR SEQ ID NO:8:

      (i)   SEQUENCE CHARACTERISTICS:
         (A)   LENGTH: 15 base pairs
         (B)   TYPE: nucleic acid
         (C)   STRANDEDNESS: single
         (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:8:

CACACACACA CACAC   15

(2)   INFORMATION FOR SEQ ID NO:9:

      (i)   SEQUENCE CHARACTERISTICS:
         (A)   LENGTH: 15 base pairs
         (B)   TYPE: nucleic acid
         (C)   STRANDEDNESS: single
         (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGTGTGTGTG TGTGT   15

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(2)   INFORMATION FOR SEQ ID NO:10:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 15 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTGTGTGTGT GTGTG   15

(2)   INFORMATION FOR SEQ ID NO:11:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 14 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCGGTTTTTT TTTT   14

(2)   INFORMATION FOR SEQ ID NO:12:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 14 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCGCAAAAAA AAAA   14

(2)   INFORMATION FOR SEQ ID NO:13:

    (i)   SEQUENCE CHARACTERISTICS:
       (A)   LENGTH: 13 base pairs
       (B)   TYPE: nucleic acid
       (C)   STRANDEDNESS: single
       (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:13:

ACACACACAC ACA   13

(2)   INFORMATION FOR SEQ ID NO:14:

    (i)   SEQUENCE CHARACTERISTICS:
       (A)   LENGTH: 13 base pairs
       (B)   TYPE: nucleic acid
       (C)   STRANDEDNESS: single
       (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGAGAGAGAG AGA   13

(2)   INFORMATION FOR SEQ ID NO:15:

    (i)   SEQUENCE CHARACTERISTICS:
       (A)   LENGTH: 13 base pairs
       (B)   TYPE: nucleic acid
       (C)   STRANDEDNESS: single
       (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:15:

TGTGTGTGTG TGT   13

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(2)   INFORMATION FOR SEQ ID NO:16:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 17 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:16:

CGGCACACAC ACACACA   17

(2)   INFORMATION FOR SEQ ID NO:17:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 21 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:17:

CTCGTAGACT GCGTACATGC A   21

(2)   INFORMATION FOR SEQ ID NO:18:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 14 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGTACGCAGT CTAC   14

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(2)   INFORMATION FOR SEQ ID NO:19:

    (i)   SEQUENCE CHARACTERISTICS:
       (A)   LENGTH: 17 base pairs
       (B)   TYPE: nucleic acid
       (C)   STRANDEDNESS: single
       (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

CTCGTAGACT GCGTACC   17

(2)   INFORMATION FOR SEQ ID NO:20:

    (i)   SEQUENCE CHARACTERISTICS:
       (A)   LENGTH: 15 base pairs
       (B)   TYPE: nucleic acid
       (C)   STRANDEDNESS: single
       (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGCTGGTACG CAGTC   15

(2)   INFORMATION FOR SEQ ID NO:21:

    (i)   SEQUENCE CHARACTERISTICS:
       (A)   LENGTH: 16 base pairs
       (B)   TYPE: nucleic acid
       (C)   STRANDEDNESS: single
       (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GACGATGAGT CCTGAC   16

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 14 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CGGTCAGGAC TCAT   14

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 17 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GGAATTCTGG ACTCAGT   17

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 21 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GATCACTGAG TCCAGAATTC C   21

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(2)   INFORMATION FOR SEQ ID NO:25:

     (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 16 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:25:

TGGCCTTTAC AGCGTC   16

(2)   INFORMATION FOR SEQ ID NO:26:

     (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 14 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:26:

TACACGCTGT AAAG   14

(2)   INFORMATION FOR SEQ ID NO:27:

     (i)   SEQUENCE CHARACTERISTICS:
          (A)   LENGTH: 17 base pairs
          (B)   TYPE: nucleic acid
          (C)   STRANDEDNESS: single
          (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTCGTAGACT GCGTACC   17

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(2)   INFORMATION FOR SEQ ID NO:28:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 17 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:28:

CTGCGTACCA GCTTACA   17

(2)   INFORMATION FOR SEQ ID NO:29:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 17 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTGCGTACCA GCTTACC   17

(2)   INFORMATION FOR SEQ ID NO:30:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 17 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:30:

CTGCGTACCA GCTTAAC   17

(2)   INFORMATION FOR SEQ ID NO:31:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 17 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:31:

CTGCGTACCA GCTTGTC   17

(2)   INFORMATION FOR SEQ ID NO:32:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 16 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:32:

CTGCGTACCA GCTTAC   16

(2)   INFORMATION FOR SEQ ID NO:33:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 16 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:33:

CTGCGTACCA GCTTAA   16

(2)   INFORMATION FOR SEQ ID NO:34:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 21 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:34:

CTCGTAGACT GCGTACATGC A   21

(2)   INFORMATION FOR SEQ ID NO:35:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 18 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:35:

GACTGCGTAC ATGCAGAC   18

(2)   INFORMATION FOR SEQ ID NO:36:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 18 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:36:

GACTGCGTAC ATGCAGAA   18

125

(2)   INFORMATION FOR SEQ ID NO:37:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 18 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GACTGCGTAC ATGCAGCA   18

(2)   INFORMATION FOR SEQ ID NO:38:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 18 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GACTGCGTAC ATGCAGTT   18

(2)   INFORMATION FOR SEQ ID NO:39:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 17 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GACTGCGTAC ATGCAGA   17

126

(2)   INFORMATION FOR SEQ ID NO:40:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 17 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GACTGCGTAC ATGCAGC   17

(2)   INFORMATION FOR SEQ ID NO:41:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 16 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GACGATGAGT CCTGAC   16

(2)   INFORMATION FOR SEQ ID NO:42:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 15 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii) MOLECULE TYPE: DNA (genomic)

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ATGAGTCCTG ACCGA   15

127

(2)　INFORMATION FOR SEQ ID NO:43:

　　　(i)　SEQUENCE CHARACTERISTICS:
　　　　　(A)　LENGTH:　17 base pairs
　　　　　(B)　TYPE:　nucleic acid
　　　　　(C)　STRANDEDNESS:　single
　　　　　(D)　TOPOLOGY:　linear

　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:43:

TGAGTCCTGA CCGAACC　　　　　　　　　　　　　　　　17

(2)　INFORMATION FOR SEQ ID NO:44:

　　　(i)　SEQUENCE CHARACTERISTICS:
　　　　　(A)　LENGTH:　17 base pairs
　　　　　(B)　TYPE:　nucleic acid
　　　　　(C)　STRANDEDNESS:　single
　　　　　(D)　TOPOLOGY:　linear

　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:44:

TGAGTCCTGA CCGAACA　　　　　　　　　　　　　　　　17

(2)　INFORMATION FOR SEQ ID NO:45:

　　　(i)　SEQUENCE CHARACTERISTICS:
　　　　　(A)　LENGTH:　17 base pairs
　　　　　(B)　TYPE:　nucleic acid
　　　　　(C)　STRANDEDNESS:　single
　　　　　(D)　TOPOLOGY:　linear

　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:45:

TGAGTCCTGA CCGACAC　　　　　　　　　　　　　　　　17

(2)   INFORMATION FOR SEQ ID NO:46:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 17 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:46:

TGAGTCCTGA CCGACAA

                                                                        17

(2)   INFORMATION FOR SEQ ID NO:47:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 16 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:47:

ATGAGTCCTG ACCGAG

                                                                        16

(2)   INFORMATION FOR SEQ ID NO:48:

        (i)   SEQUENCE CHARACTERISTICS:
              (A)   LENGTH: 16 base pairs
              (B)   TYPE: nucleic acid
              (C)   STRANDEDNESS: single
              (D)   TOPOLOGY: linear

       (ii)   MOLECULE TYPE: DNA (genomic)

       (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:48:

ATGAGTCCTG ACCGAA

                                                                        16

129

(2)   INFORMATION FOR SEQ ID NO:49:

            (i)   SEQUENCE CHARACTERISTICS:
                   (A)   LENGTH: 16 base pairs
                   (B)   TYPE: nucleic acid
                   (C)   STRANDEDNESS: single
                   (D)   TOPOLOGY: linear

           (ii)   MOLECULE TYPE: DNA (genomic)

           (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:49:

ATGAGTCCTG ACCGAT   16

(2)   INFORMATION FOR SEQ ID NO:50:

            (i)   SEQUENCE CHARACTERISTICS:
                   (A)   LENGTH: 16 base pairs
                   (B)   TYPE: nucleic acid
                   (C)   STRANDEDNESS: single
                   (D)   TOPOLOGY: linear

           (ii)   MOLECULE TYPE: DNA (genomic)

           (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:50:

ATGAGTCCTG ACCGAC   16

(2)   INFORMATION FOR SEQ ID NO:51:

            (i)   SEQUENCE CHARACTERISTICS:
                   (A)   LENGTH: 16 base pairs
                   (B)   TYPE: nucleic acid
                   (C)   STRANDEDNESS: single
                   (D)   TOPOLOGY: linear

           (ii)   MOLECULE TYPE: DNA (genomic)

           (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:51:

TGAGTCCTGA CCGAAC   16

130

(2)   INFORMATION FOR SEQ ID NO:52:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 16 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:52:

TGAGTCCTGA CCGAAA   16

(2)   INFORMATION FOR SEQ ID NO:53:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 16 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:53:

TGAGTCCTGA CCGACA   16

(2)   INFORMATION FOR SEQ ID NO:54:

    (i)   SEQUENCE CHARACTERISTICS:
      (A)   LENGTH: 17 base pairs
      (B)   TYPE: nucleic acid
      (C)   STRANDEDNESS: single
      (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:54:

GGAATTCTGG ACTCAGT   17

(2)   INFORMATION FOR SEQ ID NO:55:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 21 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:55:

GGAATTCTGG ACTCAGTGAT C   21

(2)   INFORMATION FOR SEQ ID NO:56:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 18 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:56:

TTCTGGACTC AGTGATCT .   .   18

(2)   INFORMATION FOR SEQ ID NO:57:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 18 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:57:

TCTGGACTCA GTGATCTT   18

132

(2)   INFORMATION FOR SEQ ID NO:58:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 18 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:58:

CTGGACTCAG TGATCTTC   18

(2)   INFORMATION FOR SEQ ID NO:59:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 16 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:59:

TGGCCTTTAC AGCGTC   16

(2)   INFORMATION FOR SEQ ID NO:60:

        (i)   SEQUENCE CHARACTERISTICS:
                (A)   LENGTH: 18 base pairs
                (B)   TYPE: nucleic acid
                (C)   STRANDEDNESS: single
                (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:60:

GCCTTTACAG CGTCTAAT   18

133

(2)　　INFORMATION FOR SEQ ID NO:61:

　　　　　(i)　　SEQUENCE CHARACTERISTICS:
　　　　　　　　　(A)　　LENGTH:　18 base pairs
　　　　　　　　　(B)　　TYPE:　nucleic acid
　　　　　　　　　(C)　　STRANDEDNESS:　single
　　　　　　　　　(D)　　TOPOLOGY:　linear

　　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:61:

CCTTTACAGC GTCTAATC　　　　　　　　　　　　　　　　　18

(2)　　INFORMATION FOR SEQ ID NO:62:

　　　　　(i)　　SEQUENCE CHARACTERISTICS:
　　　　　　　　　(A)　　LENGTH:　19 base pairs
　　　　　　　　　(B)　　TYPE:　nucleic acid
　　　　　　　　　(C)　　STRANDEDNESS:　single
　　　　　　　　　(D)　　TOPOLOGY:　linear

　　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:62:

CCTTTACAGC GTCTAATCA　　　　　　　　　　　　　　　　19

(2)　　INFORMATION FOR SEQ ID NO:63:

　　　　　(i)　　SEQUENCE CHARACTERISTICS:
　　　　　　　　　(A)　　LENGTH:　22 base pairs
　　　　　　　　　(B)　　TYPE:　nucleic acid
　　　　　　　　　(C)　　STRANDEDNESS:　single
　　　　　　　　　(D)　　TOPOLOGY:　linear

　　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:63:

TATATATAGA GAGAGAGAGA GA　　　　　　　　　　　　　　22

134

(2)   INFORMATION FOR SEQ ID NO:64:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 24 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:64:

ATATATATAT ATATAGAGAG AGAG   24

(2)   INFORMATION FOR SEQ ID NO:65:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 24 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:65:

CTCTCTCTCT ATATATATAT ATAT   24

(2)   INFORMATION FOR SEQ ID NO:66:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 22 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:66:

TCTCTCTCTC TCTCTATATA TA   22

(2)   INFORMATION FOR SEQ ID NO:67:

  (i)   SEQUENCE CHARACTERISTICS:
   (A)   LENGTH: 19 base pairs
   (B)   TYPE: nucleic acid
   (C)   STRANDEDNESS: single
   (D)   TOPOLOGY: linear

  (ii)   MOLECULE TYPE: DNA (genomic)

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:67:

CTCTCTCTCT CTCTCTATA   19

(2)   INFORMATION FOR SEQ ID NO:68:

  (i)   SEQUENCE CHARACTERISTICS:
   (A)   LENGTH: 20 base pairs
   (B)   TYPE: nucleic acid
   (C)   STRANDEDNESS: single
   (D)   TOPOLOGY: linear

  (ii)   MOLECULE TYPE: DNA (genomic)

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:68:

TATATATGTG TGTGTGTGTG   20

(2)   INFORMATION FOR SEQ ID NO:69:

  (i)   SEQUENCE CHARACTERISTICS:
   (A)   LENGTH: 22 base pairs
   (B)   TYPE: nucleic acid
   (C)   STRANDEDNESS: single
   (D)   TOPOLOGY: linear

  (ii)   MOLECULE TYPE: DNA (genomic)

  (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:69:

TATATATATA TATGTGTGTG TG   22

136

(2)　　INFORMATION FOR SEQ ID NO:70:

　　　　(i)　SEQUENCE CHARACTERISTICS:
　　　　　　(A)　LENGTH:　24 base pairs
　　　　　　(B)　TYPE:　nucleic acid
　　　　　　(C)　STRANDEDNESS:　single
　　　　　　(D)　TOPOLOGY:　linear

　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:70:

TATATATATA TATATATGTG TGTG　　　　　　　　　　　　　　　　　24

(2)　　INFORMATION FOR SEQ ID NO:71:

　　　　(i)　SEQUENCE CHARACTERISTICS:
　　　　　　(A)　LENGTH:　24 base pairs
　　　　　　(B)　TYPE:　nucleic acid
　　　　　　(C)　STRANDEDNESS:　single
　　　　　　(D)　TOPOLOGY:　linear

　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:71:

ACACACACAT ATATATATAT ATAT　　　　　　　　　　　　　　　　　24

(2)　　INFORMATION FOR SEQ ID NO:72:

　　　　(i)　SEQUENCE CHARACTERISTICS:
　　　　　　(A)　LENGTH:　22 base pairs
　　　　　　(B)　TYPE:　nucleic acid
　　　　　　(C)　STRANDEDNESS:　single
　　　　　　(D)　TOPOLOGY:　linear

　　　　(ii)　MOLECULE TYPE:　DNA (genomic)

　　　　(xi)　SEQUENCE DESCRIPTION:　SEQ ID NO:72:

ACACACACAC ACATATATAT AT　　　　　　　　　　　　　　　　　22

137

(2)   INFORMATION FOR SEQ ID NO:73:

       (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 20 base pairs
           (B)   TYPE: nucleic acid
           (C)   STRANDEDNESS: single
           (D)   TOPOLOGY: linear

      (ii)   MOLECULE TYPE: DNA (genomic)

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:73:

ACACACACAC ACACATATAT   20

(2)   INFORMATION FOR SEQ ID NO:74:

       (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 19 base pairs
           (B)   TYPE: nucleic acid
           (C)   STRANDEDNESS: single
           (D)   TOPOLOGY: linear

      (ii)   MOLECULE TYPE: DNA (genomic)

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:74:

TGTGTGTGTG TGTGTATAT   19

(2)   INFORMATION FOR SEQ ID NO:75:

       (i)   SEQUENCE CHARACTERISTICS:
           (A)   LENGTH: 19 base pairs
           (B)   TYPE: nucleic acid
           (C)   STRANDEDNESS: single
           (D)   TOPOLOGY: linear

      (ii)   MOLECULE TYPE: DNA (genomic)

      (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:75:

GAGAGAGAGA GAGAGATAT   19

138

(2)   INFORMATION FOR SEQ ID NO:76:

         (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 18 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

CTCTCTCACA CACACACA   18

(2)   INFORMATION FOR SEQ ID NO:77:

         (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 18 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

CTCTCTCTCA CACACACA   18

(2)   INFORMATION FOR SEQ ID NO:78:

         (i) SEQUENCE CHARACTERISTICS:
              (A) LENGTH: 18 base pairs
              (B) TYPE: nucleic acid
              (C) STRANDEDNESS: single
              (D) TOPOLOGY: linear

        (ii) MOLECULE TYPE: DNA (genomic)

        (xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

GTGTGTGTGA GAGAGAGA   18

(2)   INFORMATION FOR SEQ ID NO:79:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 18 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:79:

ACACACACAG AGAGAGAG   18

(2)   INFORMATION FOR SEQ ID NO:80:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 18 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:80:

CTCTCTCTCT GTGTGTGT   18

(2)   INFORMATION FOR SEQ ID NO:81:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 18 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:81:

AGAGAGAGTG TGTGTGTG   18

140

(2)   INFORMATION FOR SEQ ID NO:82:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 20 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:82:

TATATATGTG TGTGTGTGTG   20

(2)   INFORMATION FOR SEQ ID NO:83:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 22 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:83:

TATATATATA TATGTGTGTG TG   22

(2)   INFORMATION FOR SEQ ID NO:84:

    (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 22 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

    (ii)   MOLECULE TYPE: DNA (genomic)

    (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:84:

TATATATATA TATATATGTG TG   22

141

(2)   INFORMATION FOR SEQ ID NO:85:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 43 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:85:

TATATATATA TATATATATA TATGTGTGTG TGTGTGTGTG TGT   43

(2)   INFORMATION FOR SEQ ID NO:86:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 43 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:86:

TATATATATA TATATATATA TATCACACAC ACACACACAC ACA   43

(2)   INFORMATION FOR SEQ ID NO:87:

        (i)   SEQUENCE CHARACTERISTICS:
               (A)   LENGTH: 24 base pairs
               (B)   TYPE: nucleic acid
               (C)   STRANDEDNESS: single
               (D)   TOPOLOGY: linear

        (ii)   MOLECULE TYPE: DNA (genomic)

        (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:87:

TATATATATA TATATACACA CACA   24

(2)   INFORMATION FOR SEQ ID NO:88:

     (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 22 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:88:

TATATATATA CACACACACA CA   22

(2)   INFORMATION FOR SEQ ID NO:89:

     (i)   SEQUENCE CHARACTERISTICS:
        (A)   LENGTH: 20 base pairs
        (B)   TYPE: nucleic acid
        (C)   STRANDEDNESS: single
        (D)   TOPOLOGY: linear

     (ii)   MOLECULE TYPE: DNA (genomic)

     (xi)   SEQUENCE DESCRIPTION: SEQ ID NO:89:

TATATACACA CACACACACA   20

WHAT IS CLAIMED IS:

1. An improved method of detecting polymorphisms between two individual nucleic acid samples comprising amplifying segments of nucleic acid from each sample using primer-directed amplification and comparing said amplified segments to detect differences, the improvement comprising wherein at least one of the primers used in said amplification consists of a perfect compound simple sequence repeat.

2. The process of Claim 1 wherein said perfect compound simple sequence repeat primer is described by formula I

$$5' \ (XY)_n(NM)_n \ 3'$$
$$I$$

wherein:

n is independently 2-15;

X is A, C, T or G;

Y is A, C, T or G;
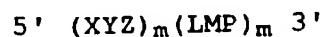
N is A, C, T or G;

M is A, C, T or G;

and provided that: X ≠ Y;

N ≠ M; and

XY ≠ NM.

3. The process of Claim 1 wherein n is independently 4 to 8.

4. The process of Claim 1 wherein said perfect compound simple sequence repeat primer is described by formula II

$$5' \ (XYZ)_m(LMP)_m \ 3'$$
$$II$$

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wherein:

        m is independently 2-10;

        X is A, C, T or G;

        Y is A, C, T or G;

        Z is A, C, T or G;

        M is A, C, T or G;

        N is A, C, T or G;

        P is A, C, T or g;

and provided that: X, Y and Z are not all the same;

                     L, M and P are not all the same;

and;

$$XYZ \neq NMP.$$

5. The process of Claim 4 wherein m is independently 2 to 4.

6. The process of Claim 1 wherein said perfect compound simple sequence repeat primer is in-phase, described by Formula III or IV

$$5' \ (XY)_n (XZ)_n \ 3'$$
$$III$$

$$5' \ (YX)_n (ZX)_n \ 3'$$
$$IV$$

wherein:

        n is independently 2-15;

        X is A, C, T or G;

        Y is A, C, T or G;

        Z is A, C, T or G;

and provided that: $Y \neq X$;

               $Z \neq X$; and

               $Y \neq Z$.

7. The process of Claim 6 wherein n is independently 4 to 8.

8. The process of Claim 6 wherein the value of n for the 5' repeating dinucleotide is greater than the value of n for the 3' repeating dinucleotide.

9. The process of Claim 6 wherein said in-phase
5 perfect compound simple sequence repeat primer is selected from the group consisting of:

$$5' \ (AC)_n(AT)_n \ 3'$$
$$(CA)_n(TA)_n$$
$$(AT)_n(GT)_n$$
10
$$(TA)_n(TG)_n$$
$$(TA)_n(CA)_n$$
$$(AT)_n(AC)_n$$
$$(TG)_n(TA)_n$$
$$(GT)_n(AT)_n$$
15
$$(TA)_n(GA)_n$$
$$(AT)_n(AG)_n$$
$$(TC)_n(TA)_n$$
$$(CT)_n(AT)_n$$
$$(AC)_n(AG)_n$$
20
$$(CA)_n(GA)_n$$
$$(CT)_n(GT)_n$$
$$(TC)_n(TG)_n$$
$$(TG)_n(AG)_n$$
$$(GT)_n(GA)_n$$
25
$$(CT)_n(CA)_n$$
$$(TC)_n(AC)_n$$
$$(AG)_n(TG)_n$$
$$(GA)_n(GT)_n$$
$$(CA)_n(CT)_n$$
30
$$5' \ (AC)_n(TC)_n \ 3'$$

wherein n is independently 2 to 15.

10. The process of Claim 1 wherein said primer-directed amplification is performed using a single primer consisting of a perfect compound simple
35 sequence repeat.

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11. The process of Claim 1 wherein said perfect compound simple sequence repeat is in-phase.

12. A process for detecting polymorphisms between two samples of nucleic acid comprising
5 separately treating each nucleic acid sample according to the steps of a-d:

a) digesting the nucleic acid with at least one restriction enzyme whereby restriction fragments are generated;

10 b) ligating adaptor segments to the ends of the restriction fragments of step a);

c) amplifying the fragments of step b) using primer-directed amplification wherein the amplification primers comprise a first primer
15 consisting of a perfect compound simple sequence repeat, and a second primer comprising a sequence which is complementary to an adaptor segment of step b); and

d) comparing the amplified nucleic acid
20 products of step c) from each nucleic acid sample to detect differences.

13. The process of Claim 12 in step c) wherein said first primer consists of a perfect compound simple sequence repeat which is in-phase.
25 14. The process of Claim 12 in step c) wherein said second primer further comprises at the 3' end from 1 to 10 arbitrary nucleotides.

15. The process of Claim 12 at step a) wherein two different restriction enzymes are used to digest
30 said nucleic acid, one restriction enzyme recognizing a tetranucleotide site on the sample nucleic acid and the other restriction enzyme recognizing a hexanucleotide site on the sample nucleic acid; and further wherein at step b) two different adaptor

segments are ligated to the restriction fragments generated at step a).

16. The process of Claim 15 wherein at step b) one of the two adaptor segments carries a member of a
5 binding pair.

17. The process of Claim 16 wherein said member of a binding pair is biotin.

18. The process of Claim 16 further comprising an additional step performed after step b):
10   b) (i) separating those fragments of step b) which carry a member of a binding pair from those fragments of step b) which do not carry a member of a binding pair; and further at step c) wherein only those fragments at step b)(i) which carry a member of
15 a binding pair are amplified according to step c).

19. The process of Claim 12 at step c) wherein said first primer carries a reporter molecule.

20. The process of Claim 19 wherein said reporter is ^{32}P or ^{33}P.
20   21. The process of Claim 12 at step c) wherein said amplification is performed using a touchdown thermocycling protocol.

22. The process of Claim 12 at step c) wherein said amplification is initiated using a hot start
25 protocol.

23. The process of Claim 13 wherein said in-phase perfect compound simple sequence repeat is selected from the group consisting of:

$$5' \ (AC)_n(AT)_n \ 3'$$
30      $$(CA)_n(TA)_n$$
$$(AT)_n(GT)_n$$
$$(TA)_n(TG)_n$$
$$(TA)_n(CA)_n$$
$$(AT)_n(AC)_n$$
35      $$(TG)_n(TA)_n$$

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$$(GT)_n(AT)_n$$
$$(TA)_n(GA)_n$$
$$(AT)_n(AG)_n$$
$$(TC)_n(TA)_n$$
5                  $(CT)_n(AT)_n$
$$(AC)_n(AG)_n$$
$$(CA)_n(GA)_n$$
$$(CT)_n(GT)_n$$
$$(TC)_n(TG)_n$$
10                 $(TG)_n(AG)_n$
$$(GT)_n(GA)_n$$
$$(CT)_n(CA)_n$$
$$(CA)_n(CT)_n$$
$$(AG)_n(TG)_n$$
15                 $(GA)_n(CT)_n$
$$(CA)_n(CT)_n$$
$$5'\ (AC)_n(TC)_n\ 3'$$

wherein n is independently 2 to 15.

24. The process of Claim 23 wherein the value of
n for the 5' repeating dinucleotide is greater than
the value of n for the 3' repeating dinucleotide.

25. A process for detecting polymorphisms
between two samples of nucleic acid comprising
separately treating each nucleic acid sample according
to the steps of a-d:

a) digesting the nucleic acid with at least
one restriction enzyme whereby restriction fragments
are generated;

b) ligating adaptor segments to the ends of
the restriction fragments of step a);

c) amplifying the fragments of step b) using
primer-directed amplification wherein the
amplification primers comprise a first primer
consisting of a simple sequence repeating region at
the 3' end and a degenerate nucleotide region at the

5' end; and a second primer comprising a sequence
which is complementary to an adaptor segment of
step b); and

      d) comparing the amplified nucleic acid

5 products of step c) from each nucleic acid sample to
detect differences.

      26. The process of Claim 25 at step c) wherein
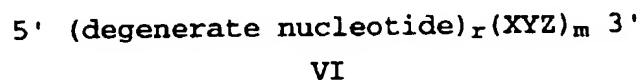said first primer is described by Formula V;

10   5' (degenerate nucleotide)$_r$(XY)$_n$ 3'
                               V
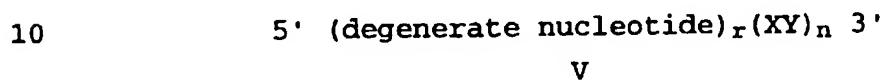
wherein:

      X is A, C, T or G;

15   Y is A, C, T or G;

      $X \neq Y$;

      r is 2 to 6; and

      n is 2 to 15.

      27. The process of Claim 25 at step c) wherein

20 said first primer is described by Formula VI;

             5' (degenerate nucleotide)$_r$(XYZ)$_m$ 3'
                           VI

25 wherein:

      X is A, C, T or G;

      Y is A, C, T or G;

      Z is A, C, T or G;

      X, Y, and Z are not all the same;
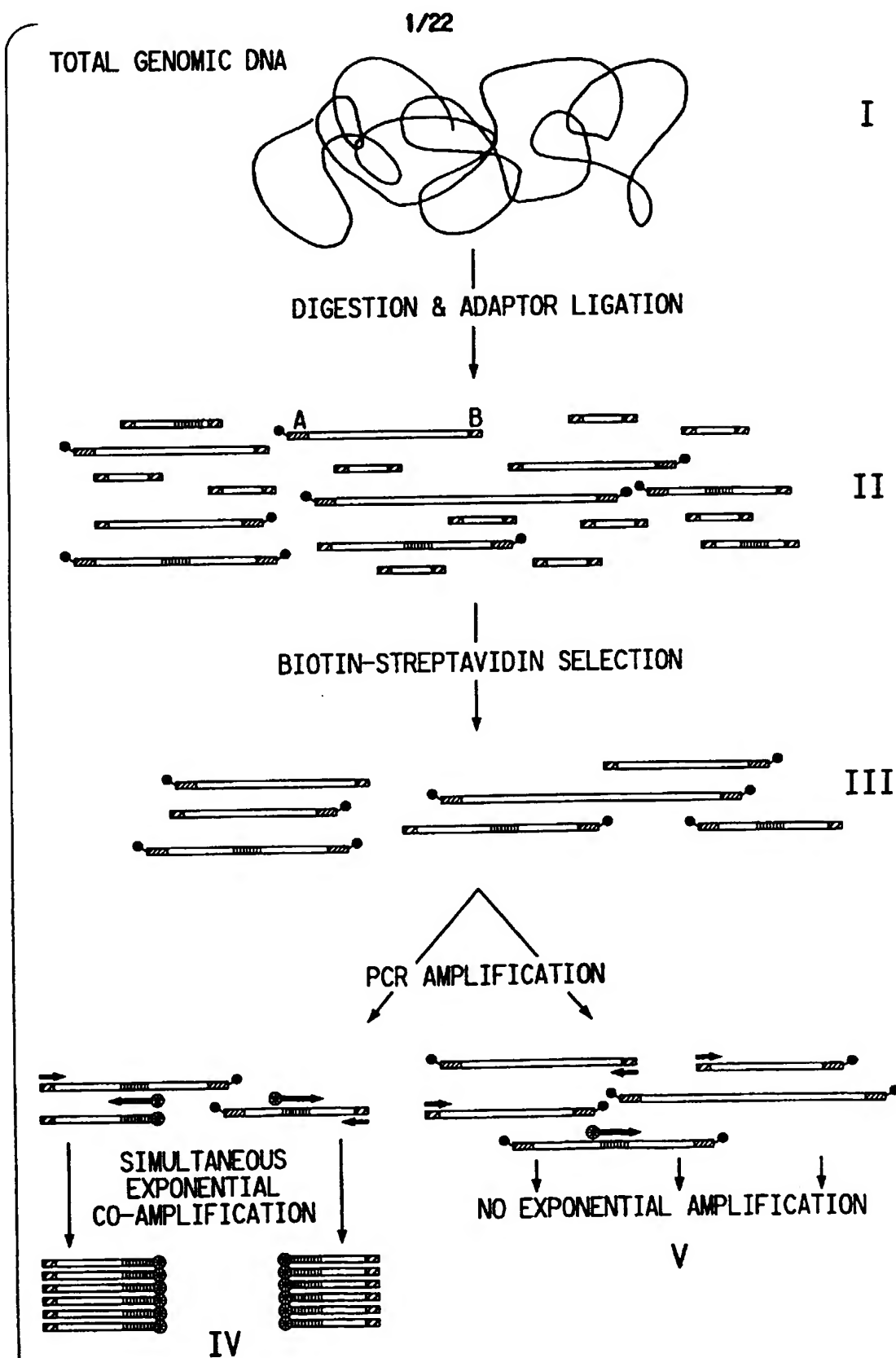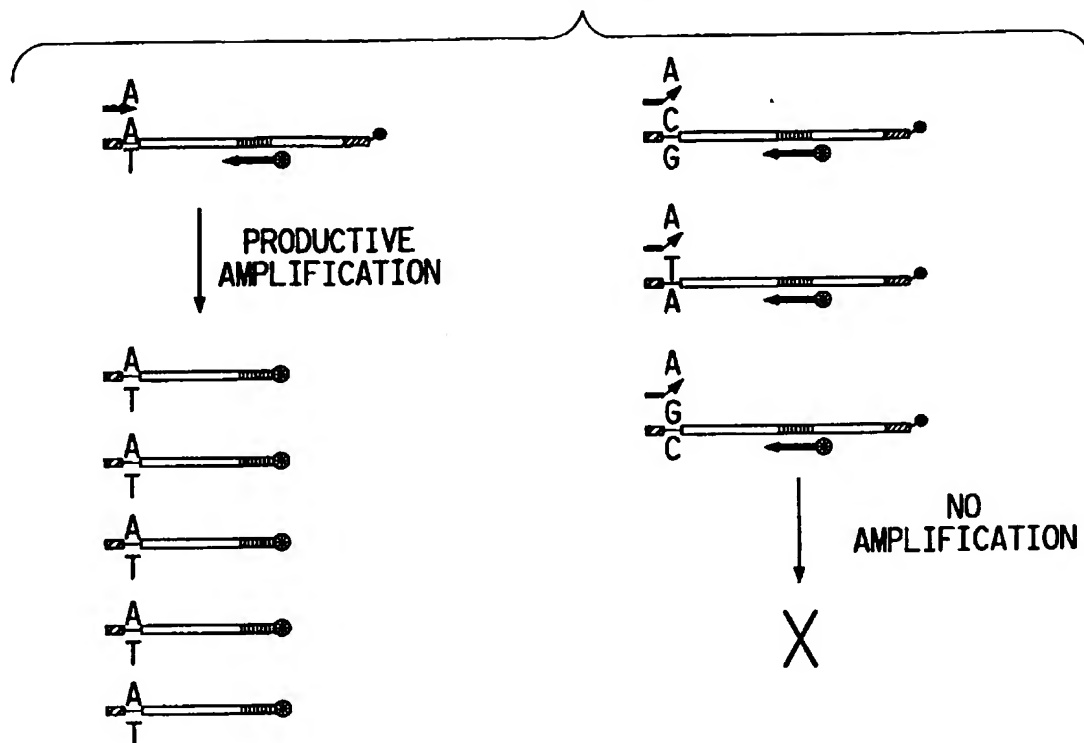
30   r is 2 to 6; and

      m is 2 to 10.

TOTAL GENOMIC DNA

I

DIGESTION & ADAPTOR LIGATION

A   B

II

BIOTIN-STREPTAVIDIN SELECTION

III

PCR AMPLIFICATION

SIMULTANEOUS
EXPONENTIAL
CO-AMPLIFICATION

NO EXPONENTIAL AMPLIFICATION

V

IV

FIG.1a

FIG.1b

PRODUCTIVE
AMPLIFICATION

NO
AMPLIFICATION

X

FIG.1c

IN-PHASE PERFECT COMPOUND SSR LOCUS: $(AT)_{11.5}(GT)_{10}$

PRIMER:

$(AT)_{2.5}(GT)_{6.5}$ TATATATGTGTGTGTGTGTG →

$(AT)_{6.5}(GT)_{4.5}$ TATATATATATATGTGTGTGTG →
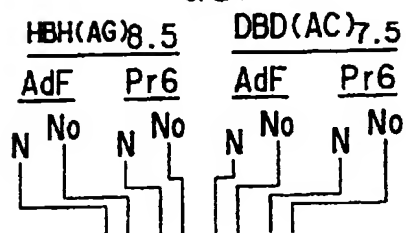
$(AT)_{8.5}(GT)_{3.5}$ TATATATATATATATGTGTG →

5'..NNTATATATATATATATATATATATATGTGTGTGTGTGTGTGTGTGTNN..3'

3'..NNTATATATATATATATATATATATCACACACACACACACACACANN..5'

$(CA)_{4.5}(TA)_{7.5}$ ← TATATATATATATACACACACA

$(CA)_{6.5}(TA)_{4.5}$ ← TATATATACACACACACACA

$(CA)_{4.5}(TA)_{7.5}$ ← TATATACACACACACACACA

SUBSTITUTE SHEET (RULE 26)

HBH(AG)8.5   DBD(AC)7.5
AdF   Pr6   AdF   Pr6
N No N No   N No N No

1100 nt ——
872 ══

603 ——

310 ——
281 ══
271 ══

◄

234 ——

194 ——

FIG.2a

118 ——

72 ——

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DBD(AC)7.5   HVH(TG)7.5

AdF		Pr6		Pr8		AdF		Pr6		Pr8	
W	S	W	S	W	S	W	S	W	S	W	S

1100 nt ——
872 ——

603 ——

310 ——
281 ==
271 ——

234 ——

194 ——

FIG.2b

118 ——

72 ——

$(TC)_{4.5}$ $(TG)_{4.5}$

TaqAd.F	Taq.pr8

2 4 6 8 10 12 14 1   3   5   7   9 11 13 15
1 | 3 | 5 | 7 | 9 | 11 | 13 | 15 | 2 | 4 | 6 | 8 | 10 | 12 | 14

$(CA)_{7.5}(TA)_{2.5}$

TaqAd.F	Taq.pr8

2 4 6 8 10 12 14 1   3   5   7   9 11 13 15
1 | 3 | 5 | 7 | 9 | 11 | 13 | 15 | 2 | 4 | 6 | 8 | 10 | 12 | 14

$(CT)_{7.5}(AT)_{3.5}$

TaqAd.F	Taq.pr8

2 4 6 8 10 12 14 1   3   5   7   9 11 13 15
1 | 3 | 5 | 7 | 9 | 11 | 13 | 15 | 2 | 4 | 6 | 8 | 10 | 12 | 14

FIG.3a

FIG.3b

$(CA)_{7.5}(TA)_{2.5}$

TaqAd.F		Taq.pr8	
P+T	H+T	P+T	H+T
2 4	2 4	5 2 x	2 4
1 3 5	1 3 5	1 3 4	1 3 5

1350 nt

1100

872

603

310

281

271

234

194

118

FIG.4

$(CA)_{7.5}(TA)_{2.5}$ + Taq.pr6
F2 INDIVIDUALS
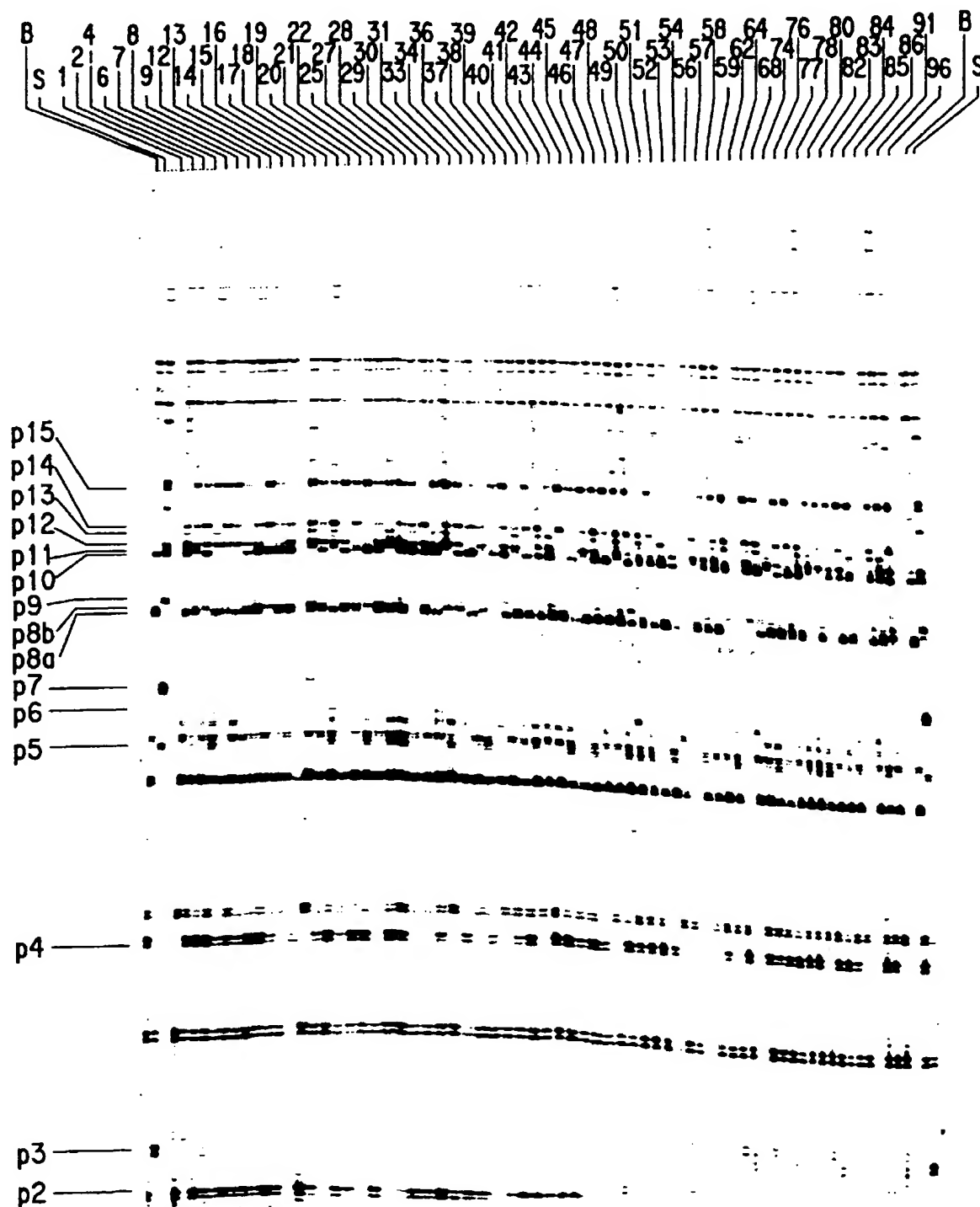
FIG.5a

FIG.5b

REC FRAC.	DIST cM	MARKER ID	NAME
		(1)	3C76
(18.3%)	22.8		
		(2)	Nod20
(18.3%)	22.8		
		(3)	460.00
(8.6%)	9.5	(4)	5503.00
(6.8%)	7.3	(5)	1076.00
(11.3%)	12.9		
(4.7%)	4.9	(6)	ALS2
		(7)	200.10
(14.4%)	16.9		
(3.7%)	3.9	(8)	902
		(9)	B71452
(9.9%)	11.0	(331)	94-23.p13rev ●
(5.8%)	6.1	(10)	1203.00
(1.5%)	1.5	(11)	p11
(1.5%)	1.5	(12)	927.00
(2.9%)	3.0	(13)	pCPGS
(4.5%)	4.7	(14)	780.00
(10.2%)	11.3	(15)	PCRBc
(8.8%)	9.7	(16)	518
(19.0%)	23.9		
		(17)	4701
(7.5%)	8.1	(18)	2309
(6.5%)	6.9	(19)	345.00
(7.7%)	8.3	(20)	892.00
(4.3%)	4.5	(21)	821.00
(5.9%)	6.3	(22)	1346.00
(14.3%)	16.9		
		(23)	410.00
(11.5%)	13.1		
		(305)	94-23.p1 ●
(11.8%)	13.4		
		(24)	4501.1

LG1

FIG.5c

REC FRAC.	DIST cM	MARKER ID	NAME
		(159)	6011
(6.9%)	7.4	(160)	3807
(7.3%)	7.9	(161)	1029.00
(8.0%)	8.8	(162)	4510
(19.5%)	24.7		
		(163)	1201.00
(13.3%)	15.5		
		(164)	618.00
(20.2%)	25.9		
		(165)	6903.10
(7.8%)	8.5	(166)	1510.00
(5.7%)	6.0	(167)	4501.2
(7.5%)	8.1	(323)	94-23.p7
(7.7%)	8.4	(168)	OLEO
(2.4%)	2.4	(169)	1148.10
(8.3%)	9.1	(170)	3415
(8.3%)	9.0		
		(171)	6411.20
(5.4%)	5.7	(172)	7320.00
(3.0%)	3.0	(173)	1351.00
(4.9%)	5.1	(174)	2311

LG10

FIG.5d

REC FRAC.	DIST cM	MARKER ID	NAME
(2.3%)	2.4	(175)	2611.00a
(3.1%)	3.2	(176)	718.00
(5.8%)	6.1	(177)	506.00
(5.3%)	5.6	(178)	169.10
		(179)	1233.00
(10.0%)	11.1		
		(180)	3217.00
(9.2%)	10.1		
		(181)	1080.00
(7.3%)	7.9		
		(182)	6006.00
(6.5%)	6.9		
		(183)	6501.00
(11.7%)	13.4		
		(184)	5719.00
(2.2%)	2.3	(185)	5220.00
(6.9%)	7.4	(186)	1461.00
(1.6%)	1.6	(187)	452.00
(11.4%)	12.9		
		(188)	4404.00
(3.0%)	3.1	(326)	94-23.p10 ●
(7.1%)	7.6	(189)	1503.00
(12.8%)	14.8		
		(307)	94-23.p5 ●
(3.8%)	4.0	(190)	1434.00
(7.9%)	8.6		
		(191)	3524.00
(17.7%)	21.9		
		(192)	5703.00

LG11

FIG.5e

REC FRAC.	DIST cM	MARKER ID	NAME
		(220)	AP8
(22.6%)	30.0		
		(221)	7108.00
(9.8%)	10.9	(324)	94-23.P8a ●
(12.6%)	14.5	(222)	621.00
(21.5%)	28.1		
(6.4%)	6.8	(223)	903.00
		(224)	3717.00
(16.5%)	20.0	(225)	7307.00
(5.7%)	6.0	(226)	UT6312
(4.0%)	4.1	(227)	6422.00
(1.6%)	1.6		
(4.2%)	4.4	(228)	4110.00
		(229)	712.00

LG14

FIG.5f

REC FRAC.	DIST cM	MARKER ID	NAME
		(230)	2623.00
(8.7%)	9.6	(231)	4809.20
(12.2%)	14.0		
(4.5%)	4.7	(232)	936.10
(5.3%)	5.6	(309)	94-23.p9 ●
		(233)	4123.00
(12.3%)	14.2		
(8.1%)	8.8	(234)	3318.00
		(235)	4905.10
(8.3%)	9.1		
(7.5%)	8.1	(236)	1000.00
(4.2%)	4.3	(237)	1209.20
		(238)	3607.20

LG15

SUBSTITUTE SHEET (RULE 26)

$(CA)_{7.5} (TA)_{2.5}$

FIG.6a

$(CT)_{7.5}\,(AT)_{2.5}$				$(GA)_{7.5}\,(TA)_2$			
TaqAd.F		Taq.pr8		TaqAd.F		Taq.pr8	
SOY	MAMMAL	SOY	MAMMAL	SOY	MAMMAL	SOY	MAMMAL

FIG.6b

$(CA)_{7.5}\ (TA)_{2.5}\ +\ Taq.pr6$

2 4 6   9

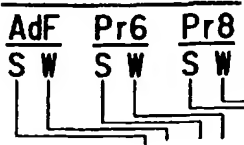1 | 3 | 5 | 7 8 |

FIG.6c

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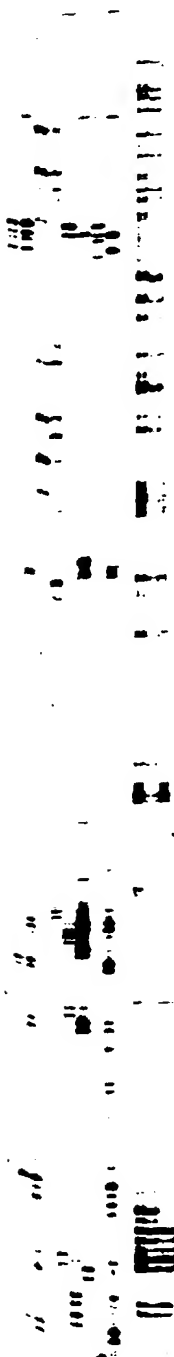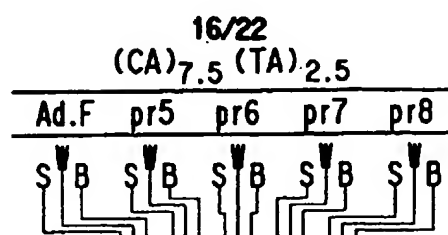
(CA)$_{7.5}$ (TA)$_{2.5}$

FIG.7
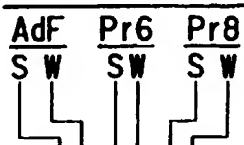
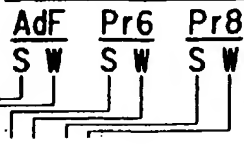$(AT)_{3.5}$ $(GT)_{6.5}$ $(CA)_{4.5}$ $(TA)_{7.5}$ $(CA)_{6.5}$ $(TA)_{4.5}$ $(CA)_{7.5}$ $(TA.)_{2.5}$

AdF Pr6 Pr8   AdF Pr6 Pr8   AdF Pr6 Pr8   AdF Pr6 Pr8
S W S W S W   S W S W S W   S W S W S W   S W S W S W

FIG.8

COLD START THERMOCYCLING   HOT START THERMOCYCLING
$(CA)_{7.5} (TA)_{2.5}$ $(CA)_{6.5} (TA)_{4.5}$ $(CA)_{6.5} (TA)_{4.5}$ $(CA)_{7.5} (TA)_{2.5}$
AdF   Pr6   Pr8   AdF   Pr6   Pr8   AdF   Pr6   Pr8   AdF   Pr6   Pr8
S W   S W   S W   S W   S W   S W   S W   S W   S W   S W   S W   S W

FIG.9

58° CONSTANT

| — | pr8 |
| — | P+T |

B S b c B S b c

58° TOUCHDOWN

| — | pr8 |
| — | P+T |

B S b c B S b c

56° TOUCHDOWN

| — | pr8 |
| — | P+T |

B S b c B S b c
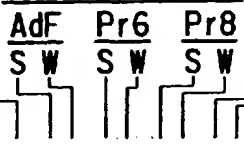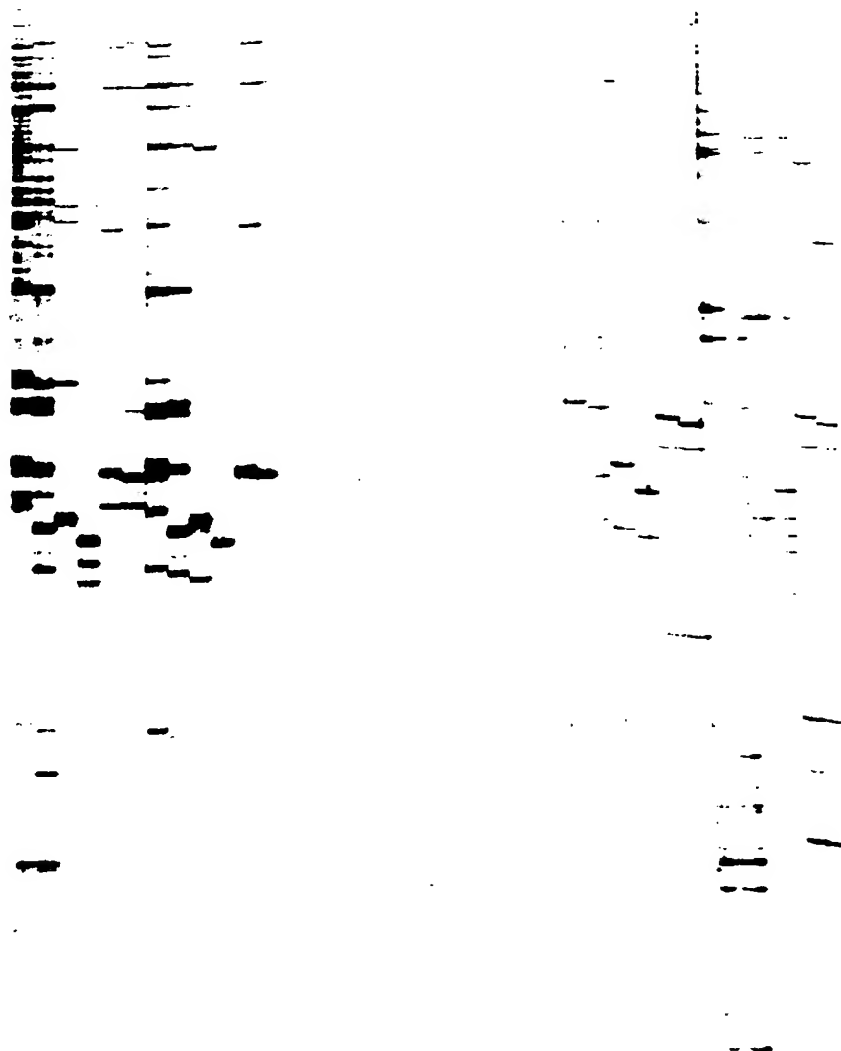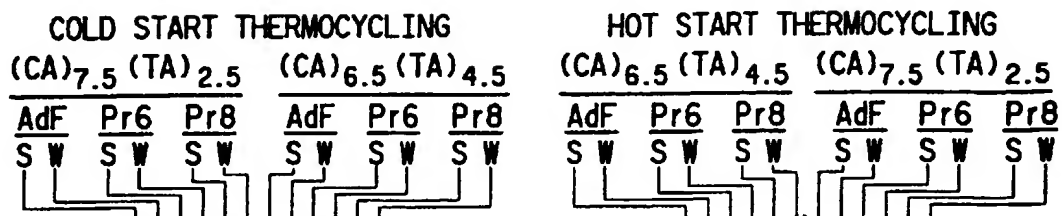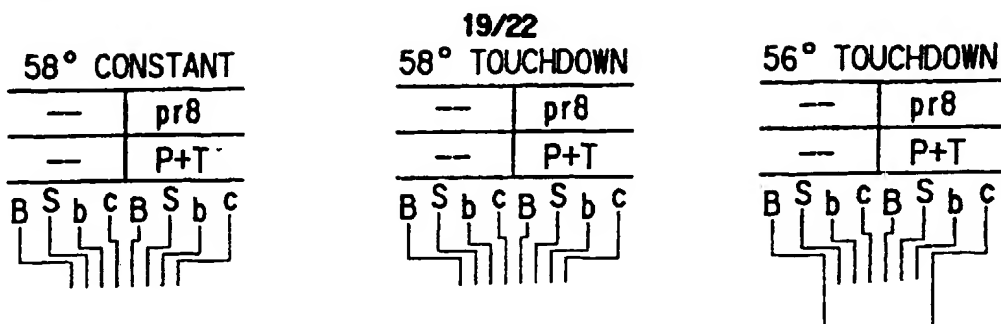
FIG.10a

20/22

CONSTANT TEMP

DBD(AC)7.5		(AT)3.5 (AG)7.5	
HBH(AG)8.5			(AT)3.5 (GT)6.5

--	P+T	--	P+T	--	P+T	--	P+T
S W	S W	S W	S W	S W	S W	S W	S W

FIG.10b

21/22
TOUCHDOWN

DBD(AC)$_{7.5}$		(AT)$_{3.5}$ (AG)$_{7.5}$	
HBH(AG)$_{8.5}$			(AT)$_{3.5}$ (GT)$_{6.5}$
--	P+T	--	P+T	--	P+T	--	P+T

S W S W S W S W S W S W S W S W

FIG.10c

FIG.11